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研究血漿 Ki-67、犬尿氨酸-3-單氧化脢和纖維蛋白原-纖維蛋白降解產物作為犬癌症診斷的生物標誌物

To Investigate Plasma Ki-67, Kynurenine 3-monooxygenase (KMO), and Fibrinogen-Fibrin Degradation Product (DR-70) As Biomarkers For Canine Cancer Diagnosis

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口試委員會審定書

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本論文係蕭嘉美君（R10629010）在國立臺灣大學獸醫學系、所完成之碩士學位論文，於民國 112 年 6 月 7 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

癌症生物標誌物被視為腫瘤診斷和預後預測的強而有力工具。這些分子由腫瘤和腫瘤微環境產生，因此存在於患者的循環系統中。腫瘤在形成過程中會引發因為組織破壞而導致止血反應的活化，進而促進腫瘤的生長和擴散。止血功能障礙常常在人類癌症患者中被檢測到，通常會導致血漿纖維蛋白原- 纖維蛋白分解產物（DR-70）水平升高。酶犬尿氨酸 3-單加氧酶（KMO）在色氨酸代謝中扮演重要之作用，先前 KMO 已被證實為神經退行性疾病和惡性腫瘤的主要標記物。Ki-67 核蛋白在細胞增殖期間增加，為腫瘤細胞進展之指標。本研究旨在評估犬癌症中 DR-70、KMO 和 Ki-67 水平的應用於犬癌診斷之可行性。本研究共蒐集 641 個臨床腫瘤犬樣本，並以 58 個健康個體作為對照組。通過酶聯免疫吸附法（ELISA）測定了血漿中 KMO、DR-70 和 Ki-67 值的濃度。結果顯示，腫瘤犬中 DR-70、KMO 和 Ki-67 的濃度明顯高於健康犬 (P<0.001)。 每個生物標誌物的 ROC (AUC) 面積分別是 DR-70 為 0.898 (P<0.001)， KMO 為 0.809 (P<0.001) ，Ki-67 為 0.533 (P<0.05)。 與單一標誌物相比，三聯合檢測的 AUC 值最高，初步判斷能有效增加腫瘤的診斷率(AUC 為 0.934)。 在這三種生物標誌物中，我們發現不同的腫瘤類型，其表現量皆顯著高於健康對照組，包括淋巴瘤、乳腺腫瘤、黑色素瘤等。此外，在同一病患在不同時間檢測 DR-70 腫瘤標誌物的案例中，我們發現 DR-70 的表現量與腫瘤進展相關，這表明其可作為追踪腫瘤發展具潛力的腫瘤標誌物。總結來說，這項研究建議 DR-70、KMO 和 Ki-67 可作為犬癌症診斷和預測腫瘤發生的生物標記物，並且預期它們在未來臨床上的應用。這是目前唯一評估血液 DR-70、KMO 和 Ki-67 在獸醫腫瘤學中作為診斷與癒後追蹤分子之研究。

中文關鍵字：纖維蛋白原- 纖維蛋白分解產物、酶犬尿氨酸 3-單加氧酶、Ki-67、犬、腫瘤、酵素聯免疫吸附法、血漿

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Abstract

Cancer biomarkers are viewed as powerful tools for tumor diagnosis and prognosis prediction. These molecules are produced by tumors and the tumor microenvironments for them could be detected in the patient's circulation. Tumor cells elicit a chronic hemostatic activation, and the pro-coagulant activities facilitate tumor growth and dissemination. Hemostatic dysfunctions are commonly detected in human cancer patients and usually result in a high plasma fibrinogen-fibrin degradation product (DR-70) level. The enzyme kynurenine 3-monooxygenase (KMO), which plays a central role in tryptophan metabolism, has previously been identified as the main factor in neurodegenerative diseases and malignant tumors. Ki-67 protein increased during cell proliferation. Therefore, this study aims to evaluate the diagnostic application of DR-70, KMO, and Ki-67 levels in canine cancers. A total of 641 clinically neoplastic canine samples and 58 healthy individuals were enrolled. The levels of plasma KMO, DR-70, and Ki-67 values were determined by the enzyme-linked immunosorbent assay (ELISA). The results showed that the expressions of DR-70, KMO, and Ki-67 were significantly increased in tumor dogs than those in healthy dogs ($P<0.001$). The determination of the area under the ROC (AUC) for each biomarker is 0.898 for DR-70 ($P<0.001$), 0.809 for KMO ($P<0.001$), and 0.533 for Ki-67 ($P<0.05$). The AUC

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value of combined detection increases the diagnostic rate compared to the single marker (AUC, 0.934). Different tumor types exhibited various levels of these three biomarkers, including lymphoma, mammary gland tumor, melanoma, etc. Furthermore, DR-70 values were correlated to tumor progression in several cases, which indicates its potential for tracing tumor development. In summary, this study suggested that DR-70, KMO, and Ki-67 levels are possible biomarkers for canine cancer diagnosis and prognosis, and their clinical application is expected which is the first study to evaluate the clinical significance of DR-70, KMO, and Ki-67 expressions in veterinary oncology.

Key words: DR-70, KMO, Ki-67, Canine, Cancer, ELISA, Plasma

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Contents

口試委員會審定書 i

誌謝 ii

中文摘要 iii

Abstract iiv

Contents vi

Chapter 1. Background and Literature Review 1

1.1 Identification and validation of cancer biomarkers 1

1.2 Canine Biomarkers in cancer cell development 1

1.3 Fibrin and Fibrinogen Degradation Products (DR-70) producing pathway 2

1.4 The mechanism, pathway, and function of KMO 3

1.5 Ki-67 as a proliferation marker in cancer 4

1.6 The clinical use of DR-70, KMO and Ki-67 in cancer patients 4

Chapter 2. Introduction 6

Chapter 3. Materials and Methods 8

doi:10.6342/NTU202302143

3.1 Study Design 8

3.2 Blood Sampling 9

3.3 Hematology and Biochemist 9

3.4 Biomarker measurement 9

3.5 Statistical Analysis 11

Chapter 4. Result 12

4.1 Patient Characteristics 12

4.2 Plasma DR-70, KMO, and Ki-67 in the tumor group are significantly higher than healthy group 13

4.3 DR-70, KMO, and Ki-67 presented in different types of cancer 13

4.4 The levels of DR-70, KMO, and Ki-67 in different conditions, including age, gender and breed, were not statistically significant in cancer patients. 14

4.5 Sensitivity and specificity of single or combined detections of plasma DR-70, KMO, and Ki-67 levels in tumor dogs 14

4.6 The expression of DR-70, KMO and Ki-67 was not statistically significant among the stages of tumor 15

doi:10.6342/NTU202302143

4.7 Cancer monitoring: DR-70 concentration proportional to tumor burden or volume.....15

Chapter 5. Discussion 17

Reference…….22

Tables……...……28

Figures…….49

doi:10.6342/NTU202302143

Chapter 1. Background and Literature Review

1.1 Identification and validation of cancer biomarkers

Cancer biomarkers can be detected in an organism's normal or abnormal biological states and can be analyzed in biomolecules such as DNA, RNA, protein, or peptide; they can be collected in body fluid, blood, urine, or tissue [1]. To identify potential cancer biomarkers in clinical practice, preclinical studies are essential. Furthermore, the development of assay and verification of candidate biomarkers depends on the analytical studies, validate the clinical assessment of biomarkers performance, and retrospect the preclinical detection capacity of cancer biomarkers, therefore the cancer control studies for evaluation of cancer burden-reducing capability of biomarkers in the population [2, 3]. The uses of cancer biomarkers include screening, differential diagnosis, risk assessment, monitoring the progression of disease, prognosis, therapeutic targets, and response to treatment [4]. In previous studies, cancer biomarkers have the potential to predict cancer development and measure the risk of tumor progression and treatment response [5].

1.2 Canine Biomarkers in cancer cell development

Cancer cell growth is uncontrol [6]. Cellular activity modifications such as apoptosis, angiogenesis, tumor suppression, cell cycle, tissue invasion, and metastasis, are the major cause of cancer cell development [7]. The cancer cell development

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procedure is gene mutation or unregular cell division and growth [8]. The regular cells genotype alteration classified normal cells as cancer cells. Cancer cell development through several pathways: cell proliferation, self-sufficient growth [9], angiogenesis, metabolism or immune modulators inhibitors [10], progression, and invasion [11]. Cancer cells and adjacent cells actively secret exosomes which are the class of extracellular vesicles in the tumor microenvironment, also promoting angiogenesis, metastasis and immunosuppression. The plasma-derived exosomes can be detected in blood samples [12]. Therefore, for the overall cancer study, we investigate the cancer biomarkers which present in cancer cell development.

1.3 Fibrin and Fibrinogen Degradation Products (DR-70) producing pathway

Fibrin and Fibrinogen Degradation Products (DR-70) is commonly used in human hemostatic dysfunction detection marker and can be detected in many cancer patients [13]. Several tumors that observed hypercoagulability [14], were related to thrombosis and associated with tumor progression. Coagulation and fibrinolysis are the pathways of cancer that elevate FDP levels [15]. In the previous study [16], the researchers indicated that cancer elevated levels by urokinase-type plasminogen activator (u-PA) and tissue factor (T.F.), cancer cells through these two pathways affect FDP production. U-PA pathway transforming plasminogen to activate plasmin, alter inactive plasmin into functional plasmin. T.F. pathway activates thrombin by changing the extrinsic

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coagulation system, and thrombin can convert Fibrinogen to Fibrin. Two substrates are digested by plasmin and can be distinguished by the type of FDP production. Fibrinogen substrates of the end products are fragments D and E, and the intermediate products are fragments X and Y. Fibrin substrates of the end products are D-dimer. Cancer cells will increase FDP levels by measuring the cancer marker DR-70 (FDP) [17], and D-dimer has been used as a diagnostic tool for coagulopathies and thrombotic disease, also screening cancer [18].

1.4 The mechanism, pathway, and function of KMO

Kynurenine 3-monooxygenase (KMO) is one of the critical enzymes in the kynurenine pathway (K.P.) [19], it is the central route of tryptophan (Trp) metabolism [20], and it will cause several toxic metabolites for the response of the neurodegenerative, inflammatory disorders, depression, schizophrenia, autoimmunity, and cancer [21]. In the K.P. pathway, three rate-limiting enzymes catabolized tryptophan into kynurenine, which is tryptophan 2,3-dioxygenase (TDO) in the liver and indoleamine 2,3-dioxygenase 1/2 (IDO1/2) in peripheral tissues [22], both will increase kynurenine by stress hormones or inflammatory factors, and the role of IDO/TDO has been intensely investigated in many types of cancer [23]. KMO, located on the outer membrane of mitochondria, and can be detected on the cell membranes [24], catalyzes the conversion of kynurenine into 3-hydroxykynurenine (3-HK), 3-HK

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is converted to 3-hydroxy anthranilic acid (3-AA), and converted to quinolinic acid (Q.A.) [25], picolinic acid, nicotinamide adenine dinucleotide (NAD+), and other molecules. KMO levels are elevated in many types of cancer, such as human breast cancer [26] and colorectal cancer [27]. Also, KMO expression will increase in canine mammary gland tumor [28] and melanoma [29] patients.

1.5 Ki-67 as a proliferation marker in cancer

The Ki-67 protein marker is a vital proliferation marker used in pathology [30]. In the regulation of the cell cycle of Ki-67, it is present in every active phase of the cell cycle except the G0 phase or resting period [31]. Ki-67 levels will decrease sharply in the later stages of mitosis [32]. The Ki-67 protein expression will increase with the proliferating cancer cell in malignant tumors and as a marker of tumor aggressiveness [33].

1.6 The clinical use of DR-70, KMO and Ki-67 in cancer patients

In previous studies, these three cancer biomarkers have been used in different types of human cancer prediction, detection, and prognosis. DR-70 has been used as a screening and diagnostic tool in coagulopathies and thrombotic disease [34]; hemostatic abnormalities that are closely related to cancer have been demonstrated [35]. DR-70 has high expression in many malignancies, such as colorectal carcinoma (CRC) [36], hepatocellular carcinoma (HCC) [37], and cholangiocellular carcinoma (CCC) [13], in

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human clinical studies. KMO is an enzyme in the K.P. pathway, and it reported the level of KMO expression is high in neurodegenerative [38], inflammatory disorders [39] and cancer. In human clinical studies, KMO overexpressed in breast cancer patients, particularly in triple-negative breast cancer (TNBC) [40]; we also demonstrated KMO values increased in canine mammary gland tumors in previous studies [28]. Ki-67 has been found in different types of cancer as a tumor-aggressive marker [41] in malignant tumors, such as human breast cancer [42], canine melanoma [43], colorectal cancer [44], etc. Therefore, DR-70, KMO, and Ki-67 have been widely used in human cancer clinical studies but not in canine cancer as tumor detection biomarkers.

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Chapter 2. Introduction

The use of cancer biomarkers stands as a big challenge of oncology in both human and veterinary medicine. An ideal tumor biomarker would be easy to measure, have the perfect sensitivity and specificity, and differentiate individuals from healthy individuals and the neoplastic process at the earliest possible cancer stages [45]. It would also be able to detect and monitor cancer development or predict relapses. The cancer biomarkers should identify the patient affected by any tumor process [46].

Many studies demonstrated that the combined detection of biomarkers can elevate the diagnostic rate of tumors [47-49]. DR-70 is a fibrinogen fibrin degradation product, which is not only related to thrombosis but also associated with tumor progression [50]. DR-70 has been used as a screening and diagnostic tool in numerous coagulopathies, thrombotic diseases [51] and screening of cancers, it can be detected in different organs type of human cancer. Kynurenine 3-monooxygenase (KMO) is an enzyme that relies on dietary tryptophan and is a key site in the kynurenine pathway [52]. The kynurenine pathway decreases inflammation in inflammatory disease and prolongs the survival of cancer cells [53]. In our laboratory's previous study, KMO transcription level is significantly upregulated in human breast cancer [40], and KMO gene expression is significantly higher in the malignant group of canine mammary gland tumors compared with benign group patients [28]. Ki-67 is a nuclear protein that is present in all active

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phases (G1, S, G2 and mitosis) in the cell cycle except resting cell (G0) [54], and is commonly used as a proliferation marker in human breast cancer.

The present study aims to clarify whether DR-70, KMO and Ki-67 in plasma that can be the biomarker in canine cancer detection. The cancer biomarkers level between healthy control and tumor patients, and the expression among the tumor types and cancer stages. The multiple specific tumor markers combined detection to improve the diagnostic rate of canine tumors, and also can be able to predict the development of cancers.

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Chapter 3. Materials and Methods

3.1 Study design

All samples were obtained from canine patients who were admitted to National Taiwan University Veterinary Hospital (NTUVH) from 2005 to 2022. The retrospective cohort of plasma samples collected 641 cancer patients between the age of 1 to 18 years old (median was 10 years old), including lymphoma, mast cell tumor, melanoma, mammary gland tumor, transitional cell carcinoma, and perianal gland tumor. Patients were diagnosed by the method of fine needle aspiration (FNA), cytology, histopathology, flow chemistry, x-ray, or ultrasound. Dogs with tumors have abnormal growth of cells, abnormal undifferentiated cells found by cytology, bizarre tumor invasion look under a microscope of histopathology, anomalous lymphocyte subset of flow cytometry, abnormal enlargement of lymph node or tumor mass under x-ray or ultrasound [55] . The 58 Ethylenediamine tetraacetic acid (EDTA) blood samples from 2021 to 2022 healthy donors plasma sample were used as a negative control, including tumor-free and inflammation-free, routine complete blood count (CBC) and serum biochemical analysis canine, routine body check and regular deworming, absence of systemic illness [56]. This study was approved by the Institutional Animal Care and Use Committee of the National Taiwan University (Approval No: IACUC No. NTU110-EL-00096).

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3.2 Blood sampling

In two weeks, the maximum blood volume that can be collected may not exceed 1 % of the dog's body weight (or 10 ml/kg) [57]. The 3 mL blood sample was collected from each dog via canine peripheral blood in EDTA tube (367835, BD Vacutainer), 1500 rpm, 4°C for 5 min centrifuge and collected supernatant into 1.7 ml Eppendorf, after centrifuge 16,000 g, 4°C for 10 min, collected plasma sample into a new 1.7 ml Eppendorf, stored at -80° C until analyzed.

3.3 Hematology and Biochemist

Blood samples were collected by canine vessel blood using disposable syringes (3 mL with needle; NIPRO). Used EDTA tube collected 3 mL blood for CBC count, and the heparin tube collected 7.5 ml for serum separation. CBC count using an automated veterinary hematology analyzer (ProCyte Dx; IDEXX) [58]. Heparin tubes (367886; BD Vacutainer) were centrifuged at 1800 g for 10 min, collected serum to Eppendorf, and stored at -20°C until analyzed. The amounts of alkaline phosphatase (ALP), Aspartate aminotransferase (AST), total bilirubin (Bil), blood urea nitrogen (BUN), creatinine (CRE), calcium (Ca) were measured by commercial kits using an autoanalyzer (0-JJ-VITROS350; Ortho Clinical Vetros 350) [59].

3.4 Biomarker measurement

According to a previous study [60], combined detection of biomarkers in serum or

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plasma may improve the detection sensitivity of tumors, providing reference value for clinical application. Three double antibody sandwich enzyme-linked immunoassay (ELISA) kits were used for this study: DR-70 (DR2101; Huayu Taiwan), KMO (MBS8803176; MyBioSource) and Ki-67 (MBS089640; MyBioSource) ELISA kit. Samples and standard curves were run in duplicate. The procedures should be carried out according to the ELISA kit recommendation of the manufacturers, respectively. DR-70 and KMO biotin-conjugated antibodies coated on the microtiter plate with 100 µl of plasma samples and standard buffer for each well (The canine plasma was diluted 1:250 in DR-70 and 1:20 in KMO); subsequently, it incubated at room temperature for 30 mins of DR-70, at 37ºC for 80 mins of KMO; After 5 washes with wash buffer added 100 µl avidin conjugated to Horseradish Peroxidase (HRP) and incubated at room temperature for 30 mins of DR-70, or at 37oC for 50 mins of KMO; After 3 washes with wash buffer, added 100 µl Tetramethyl Benzidine (TMB) incubated at room temperature for 15 mins of DR-70, at 37ºC for 20 mins of KMO. The Ki-67 biotin-conjugated antibodies were coated on the microtiter plate with 50 µl of undiluted original canine plasma samples and standard buffer for each well, after adding 50 µl HRP in every well, incubated at 37ºC for 60 mins; After 4 washes with wash buffer, added 100 µl TMB, and incubated at 37ºC for 15 mins. The reaction of the intensity was terminated of optical density (O.D.) by a microplate reader at 450 nm. The plasma

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concentrations of DR-70, KMO, and Ki-67 were obtained from a standard curve. The detection limits of these three ELISA kits were DR-70 (0.14-5.2 μg/mL), KMO (0.79-50 ng/mL), and Ki-67 (0.625-20 ng/mL).

3.5 Statistical analysis

Statistical analysis was performed using Prism 9.0 software (GraphPad). The data were described as the mean ± interquartile range (IQR). Mann-Whitney U test was applied to compare the variables between tumor-free and tumor groups, and the Kruskal-Wall test of One-Way ANOVA was used to observe the variety of tumor types. $P < 0.05$ was considered statistically significant. For each cut-off, sensitivity, specificity, and the area under the curve (AUC) with a 95% confidence interval (Cl) of tumor biomarkers in the diagnosis of cancer canine, the tumor marker was defined by the method of ROC curve. The higher the area under the curve (AUC) represents a higher diagnostic value; the ideal index of AUC is 1.0, and if AUC <0.5 is no diagnostic value [61].

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Chapter 4. Result

4.1 Patient characteristics

We collected a total of 699 plasma samples from 2005 to 2023, out of which 58 were obtained from healthy donors, and 641 were tumor samples that were included in the study. These tumor plasma samples were further divided into 114 cases collected from other animal hospitals and 527 tumor cases from National Taiwan University Animal Hospital (NTUAH). Among them, consecutive samples from the same patient at different time points accounted for 239.

According to Table 1, in NTUAH tumor samples, 288 tumor patients were subjected to experimental statistics. The median age was 10 (age range 1 to 18 years, mean, 10 years old). Gender distribution was 133 male and 155 female. Over than twenty breeds were presented, 88 mixed, 29 poodles, 27 schnauzers, 25 Golden retrievers, 18 Maltese, 14 Dachshunds, 11 Beagle, 9 Border Collies, 9 French Bulldog, 8 Shiba Inu, 6 Welsh Corgis, 6 Husky, and 41 of other breeds.

According to Table 2, among the type of tumor, 68 dogs with lymphoma, 28 dogs with mast cell tumor, 28 dogs with sarcoma, 27 dogs with carcinoma, 17 dogs with melanoma, 15 dogs with mammary gland tumor, 15 dogs with adenocarcinoma, 13 dogs with transitional cell carcinoma, 6 dogs with squamous cell carcinoma. The tumor stage was determined according to the WHO TNM system.

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4.2 Plasma DR-70, KMO, and Ki-67 in tumor group are significantly higher than healthy group

Between the healthy and tumor plasma samples, we found that the tumor plasma samples of DR-70 mean was 2.22 ($P< 0.0001$), KMO mean was 2.03 ($P< 0.0001$), and Ki-67 mean was 4.21 ($P< 0.05$) levels in the tumor group were significantly higher than that in the healthy group, which mean was 1.39 in DR-70, 0.84 in KMO, and 3.86 in Ki-67 (Figure 1). These three markers could be used to distinguish between cancer patients and healthy dogs.

4.3 DR-70, KMO, and Ki-67 presented in different types of cancer

The results showed that DR-70, KMO, and Ki-67 presented in different cancer types, which were significantly higher than the healthy control group ($P<0.005$), including lymphoma, mast cell tumor, melanoma, mammary gland tumor, transitional tumor cell carcinoma, and squamous cell carcinoma. According to Table 2, we found that squamous cell carcinoma has a higher level of DR-70, the mean was 2.26± 0.55, and in sarcoma, the mean was 2.26± 0.58. KMO has a higher value in mammary gland tumors, the mean was 4.41± 5.59, and a higher concentration of Ki-67 has expressed in mammary gland tumors, the mean was 4.22± 1.14, and also in melanoma, the mean was 4.21± 1.41. There was no statistically significant among cancer types ($P< 0.573$). (Figure 2)

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4.4 The levels of DR-70, KMO, and Ki-67 in different conditions, including age, gender, and breed, were not statistically significant in cancer patients.

In clinical studies, investigating the states which have affected the cancer biomarker concentration is essential. According to Table 3, among the tumor patients, the result showed that there were no statistically significant differences in age, gender, and breed. (Figure 3,4,5) It demonstrates that age, gender or breed will not affect the concentration of these three biomarkers, this result is very important for the early detection of cancer patients.

4.5 Sensitivity, specificity of single and combined detections of plasma DR-70, KMO, and Ki-67 levels in tumor dogs

According to the ELISA results of Table 4, we collected 180 patients tested simultaneously for three biomarkers and 58 healthy controls for comparison to assess diagnostic accuracy. The validation showed that the AUC of DR-70 in tumor patients was 0.898, with 82.8 % sensitivity and 79.4 % specificity; The AUC of plasma KMO was 0.809 in tumor patients, with 82.8 % sensitivity and 65.6 % specificity; The AUC of plasma Ki-67 was 0.533, with 67.2 % sensitivity and 46.1 % specificity (Figure 6a). Among two cancer biomarkers, combined detection of plasma DR-70 and KMO presented the AUC was 0.899, with 72.4 % sensitivity and 90.6 % specificity; DR-70 and Ki-67 were 0.932, with 94.8 % sensitivity and 80 % specificity; KMO and Ki-67 were 0.834, with 98.3 % sensitivity and 61.1 % specificity (Figure 6b). Three cancer

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biomarkers combined detection of plasma DR-70, KMO, and Ki-67 presented the AUC as 0.934, with 100 % sensitivity and 74.4 % specificity (Figure 6c). The AUC value of combined detection will increase the diagnostic rate of single or two markers detection.

4.6 The expression of DR-70, KMO, and Ki-67 was not statistically significant among the stages of the tumor

For the clinical study, we found that the level of DR-70, KMO, and Ki-67 in different stages of the tumor, there was no statistically significant among the stages of cancer, but the result showed that the late-stage level of DR-70 value is high than the early-stage. (Figure 7)

4.7 Cancer monitoring: DR-70 concentration proportional to tumor burden or volume

In NTUAH serial cases, we found 26 tumor patients, 8 of lymphoma, 7 of B cell lymphoma, 5 of mast cell tumor, 2 of transition cell carcinoma, 2 of squamous cell carcinoma, 1 of nasal adenocarcinoma, and 1 of prostate tumor. The result showed that 12 tumor patients DR-70 value proportional to tumor burden of volume, and 8 tumor patients DR-70 concentration changed but tumor changes were maintained. The stage 4b of B cell lymphoma dog, DR-70 expression increased when the tumor was diagnosed as a progressive disease (PD), and decreased when the tumor size was diagnosed as a complete response (CR) (Figure 8a). In another B cell lymphoma case, we discovered that when tumor size was diagnosed as a partial response (PR), DR-70 had a different

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level, we believed that various interfering could change the level of biomarkers in the tumor microenvironment. The same as the previous clinical results, DR-70 expression increased when the tumor was or size diagnosed as progressive disease, and decreased when tumor size was diagnosed as stable disease (SD). (Figure 8b). In addition, squamous cell carcinoma had the same result, when the tumor size was diagnosed as a progressive disease, DR-70 expression increased (Figure 8c). In another squamous cell carcinoma case, we found the same result (Figure 8d). In these serial cases result, we suggest DR-70 can be a cancer biomarker to predict and monitor the development of tumors.

Three lymphoma patients and one mast cell tumor patients had DR-70 expression changes but the tumor size was maintained (Figure 8 e, f, g, h). However, we need more clinical samples for future research.

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Chapter 5. Discussion

In this study, we suggested that DR-70, KMO, and Ki-67 are potential biomarkers for canine cancer diagnosis and prognosis in clinical applications. The level of cancer biomarkers varies during the disease occurs and develops [62]. These molecules are present in tumor tissues and patient body fluids [63] for us to monitor them in a low-invasive method. Their concentration can identify the presence of tumors even during the early development stage of cancer before imaging and other diagnostic methods are revealed [64, 65]. However, using a single biomarker is usually non-specific, and the sensitivity and specificity are also varied [66]. Therefore, the detection of multiple cancer biomarkers should increase diagnostic accuracy.

We compared the different expression of DR-70, KMO, Ki-67 among age, gender and breed, the result showed that there were not statistically significant. In our patients list of Table 1, we found that 35/288 cancer patients were younger than 7 years old, tumor can occur in any age, any gender and any breed. It is very important for the early detection of canine cancer patients.

Hypercoagulability is often found in several tumors and is related to thrombosis and tumor progression. D-dimer, the fibrinogen-fibrin degradation product is also frequently observed in blood disorders caused by inflammation. C-reactive protein (CRP) is the acute-phase protein and is an exquisitely sensitive marker of inflammation

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and tissue damage [67]. The acute-phase response includes tissue damage, infection, inflammation, and malignant neoplasia [68]. To distinguish the causes for the rise of DR-70 levels among healthy dogs, cancer dogs, and dogs with inflammation, we collected 55 plasmas from healthy donors, 97 tumor plasmas, and 11 dogs with inflammation to detect the concentration of CRP and DR-70. We found that CRP levels have no statistical significance between the group of healthy dogs and tumor dogs (P= 0.7957), healthy dogs and dogs with inflammation (P= 0.2342), tumor dogs and dogs with inflammation (P= 0.0752). (Figure 9). Compared to the healthy controls, the DR-70 levels were significantly higher in tumor (P< 0.0001) and inflammatory dogs (P< 0.0223), but no statistical significance between dogs will inflammation and tumor dogs (P= 0.8848). (Figure 10). In the previous studies, recent data extend the notion that inflammation is a key component of tumor progression. Many cancers arise from chronic irritation, site of infection, and inflammation, caused by the tumor microenvironment [69], orchestrated primarily by the inflammatory cell which is a participant in the cancer process, fostering, proliferation, survival and migration [70]. This result suggests that DR-70 can be used for canine cancer screening but CRP maybe cannot. The plasma half-life of CRP is very short (about 19 hours in dogs) [71], and the stage of inflammation also needs to be considered, we need more samples as follow-up proof.

doi:10.6342/NTU202302143

In this study, DR-70 can be detected in different types of cancer, including lymphoma, mast cell tumor, adenocarcinoma, melanoma, mammary gland tumor, transitional cell carcinoma, perianal gland tumor and squamous cell carcinoma, the highest level of DR-70 is squamous cell carcinoma (2.26 ± 0.55), this result is consistent with previous research [72]. The biomarkers levels in different stages of cancers were not statistically significant among the stage of the group (*P*= 0.1391), but late-stage cancer has a higher concentration of DR-70. In some serial cases, we found that the concentration of DR-70 is positively correlated with the size of the tumor in lymphoma and squamous cell carcinoma patients. This result suggests DR-70 can be used in screening and predicting the development of tumors. KMO is recognized as a tumor marker for the diagnosis of human breast cancer. In our laboratory previous research that the KMO gene overexpressed in the malignant canine mammary gland tumor. In this study, levels of KMO can be detected in different types of cancers, we found that KMO level was the highest in canine mammary gland tumors (mean is 4.41 ± 5.59), and was significantly higher than healthy controls (P<0.0001), this result is consistent with previous research [28], but there were no statistically significant among the stage of cancer (*P*=0.9023). The sensitivity and specificity of KMO were 82.8 % and 65.6 %. Clinical studies have shown that Ki-67 is related to cell progression, a higher concentration of Ki-67 can be detected in human breast cancer and also in canine

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mammary gland tumors. In the present study, the level of Ki-67 in tumor patients was significantly higher than that of the healthy control group (P<0.05). We found that Ki-67 level can be detected in many types of cancer, the highest concentration is mammary gland tumor (4.22 ± 1.14). The result is consistent with previous research [41], but there were no statistically significant among the stage of cancer (P= 0.6636). The sensitivity and specificity of Ki-67 were 67.2 % and 46.1 %. For the low sensitivity and specificity of Ki-67, combined detection with other cancer biomarkers is required.

This study demonstrated that combined detection of DR-70, KMO and Ki-67 improves diagnostic accuracy and has a higher sensitivity than the double and single cancer biomarker detection. In ROC curve analysis, the area under the ROC curve of DR-70 single detection is better than KMO and Ki-67. Among the double cancer biomarkers detection, the highest AUC value is DR-70+ Ki67, which was 0.932, DR-70+ KMO was 0.899 as follows, and the lower group is KMO+ Ki67, which was 0.834. Three biomarkers combined detection has the highest diagnostic rate, AUC was 0.934. The result suggests that multiple biomarkers combined detection can increase the diagnostic rate in canine cancer diagnosis.

The detection limits of commercial enzyme-linked immunosorbent assay (ELISA) are the sensitivity and the affinity between the antigen and antibody of the target protein. The probably reason of the lowest AUC value of Ki-67 is that because Ki-67 a nuclear

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protein, it is unclear whether using plasma will affect the detection value. The disadvantage of using ELISA for detection is that it needs to be sent to the laboratory for detection, and the value cannot be measured immediately the in the animal hospital. Therefore, the research and development of cancer biochip detection can provide real-time and low-invasive cancer detection in the future.

In conclusion, this study demonstrated the plasma cancer biomarkers DR-70, KMO and Ki-67 can be used as diagnostic tools for screening canine tumors, the cancer biomarkers of cancer patients were significantly higher than healthy control and the biomarkers concentration will not change among the group of age, gender and breed. The combined detection of DR-70, KMO and Ki-67 increases the sensitivity and diagnostic accuracy. This is the first time that combined detection of these three cancer biomarkers in the diagnosis of canine tumors in veterinary oncology. The results suggest that the multiple cancer biomarkers combined detection of DR-70, KMO and Ki-67 for canine cancer diagnosis and prognosis, the cancer biochip development of the clinical canine cancer diagnosis can be one of the regular annual health check projects in the future.

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Tables

Table 1 Summarize tumor patient characteristics from NTUAH

No.	Species	Breed	Gender	Age	Tumor type	Stage
1	Canine	Schnauzer	F	10	Melanoma	
2	Canine	English Bulldog	F	9	B cell lymphoma	IV
3	Canine	Mixed	M	14	Transitional cell carcinoma	
4	Canine	Border Collies	M	10	Unknown	
5	Canine	Border Collies	F	8	Mammary gland tumor	V
6	Canine	French Bulldog	F	6	Transitional cell carcinoma	I
7	Canine	Border Collies	F	14	Transitional cell carcinoma	
8	Canine	French Bulldog	M	4.5	B cell lymphoma	IV
9	Canine	Mixed	F	11	Osteosarcoma	V
10	Canine	Mixed	F	8	Lymphoma	
11	Canine	Poodle	F	7	T cell lymphoma	
12	Canine	Maltese	Fsp	10	maxillary melanoma	
13	Canine	Beagle	M	16	perianal gland tumor	
14	Canine	Mixed	Fsp	4	plasmacytoma	
15	Canine	Mixed	M	9	Hind limb paraparesis	

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16	Canine	Mixed	M	10	T cell lymphoma	
17	Canine	Schnauzer	M	14	Melanoma	III
18	Canine	Mixed	M	4	T cell lymphoma	
19	Canine	Mixed	F	9	Mammary gland tumor	
20	Canine	Mixed	F	5	Multicentric lymphoma	V
21	Canine	Mixed	F	4	Solitary osseous plasmacytoma	
22	Canine	Welsh Corgi	F	2	Osteosarcoma	
23	Canine	Welsh Corgi	F	8	T10 intramedullary spinal tumor	
24	Canine	Mixed	F	14	Intestinal T cell lymphoma	
25	Canine	Border Collies	M	7	T cell lymphoma	
26	Canine	Mixed	F	9	Multicentric lymphoma	V
27	Canine	Mixed	F	11	Lymphocytosis: leukemia CLL	
28	Canine	Border Collies	M	Unknown	Transitional cell carcinoma	I
29	Canine	Poodle	M	10	Oral melanoma	IV
30	Canine	Schnauzer	Mc	Unknown	Mast cell tumor	I
31	Canine	Border Collies	F	7	Mast cell tumor	
32	Canine	Schnauzer	M	14	Oral melanoma	III
33	Canine	Poodle	M	8	Mast cell tumor	II
34	Canine	Golden retrievers	M	8	Histiocytic sarcoma	
35	Canine	Mixed	M	7	Panniculitis	

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36	Canine	Mixed	M	12	Chronic lymphocytic leukemia	
37	Canine	Poodle	M	6	B cell lymphoma	
38	Canine	Maltese	F	14	Transitional cell carcinoma	V
39	Canine	Terrier	F	5.5	Renal cell carcinoma	V
40	Canine	Maltese	M	12	T cell lymphoma	
41	Canine	Border Collies	F	8	Mammary gland tumor	
42	Canine	Beagle	M	9	IMHA, adenocarcinoma	
43	Canine	Shiba Inu	F	10	B cell lymphoma	V
44	Canine	Schnauzer	M	12	Small	I
45	Canine	Japanese Chin	F	15	T cell CLL	
46	Canine	Mixed	F	8	Histiocytic sarcoma	IV
47	Canine	Mixed	Mc	Unknown	Mast cell tumor	I
48	Canine	Schnauzer	M	10	Oral melanoma,	III
49	Canine	Husky	F	11	Mammary gland tumor	
50	Canine	Poodle	F	5	Unknown	
51	Canine	Mixed	F	9.5	Thyroid tumor	
52	Canine	Maltese	M	13	Splenic leiomyosarcoma	
53	Canine	Mixed	F	6	Mast cell tumor	IV
54	Canine	Mixed	F	5	Mast cell tumor	II
55	Canine	Schnauzer	M	12	Nasal planum melanoma	

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56	Canine	Shiba Inu	F	5	Lymphoma	V
57	Canine	Schnauzer	F	11	Squamous cell carcinoma	II
58	Canine	Mixed	F	8	Digit melanoma	II
59	Canine	Mixed	M	6	Lymphoma	IV
60	Canine	Mixed	M	15	UB mass	
61	Canine	Poodle	F	11	Transitional cell carcinoma	II
62	Canine	Golden retrievers	M	13	Sarcoma	IV
63	Canine	Dachshunds	F	13	Multicentric lymphoma	III
64	Canine	Mongrel	F	unknown	Anal sac adenocarcinoma	III
65	Canine	Poodle	F	4	Lymphoma	IV
66	Canine	French Bulldog	M	10	Nasal osteosarcoma, GI lymphoma	III
67	Canine	Dachshunds	M	unknown	Prostate tumor	
68	Canine	Labrador	F	5	Rt anal sac adenocarcinoma	II
69	Canine	Dachshunds	M	7	Mast cell tumor	
70	Canine	Mixed	M	17	Liver tumor	III
71	Canine	Schnauzer	F	10	B cell lymphoma	IV
72	Canine	Dachshunds	F	8	Mammary gland tumor	III
73	Canine	Terrier	M	6	B cell lymphoma	IV
74	Canine	Afghan Hound	M	6	Perianal gland carcinoma	
75	Canine	whippet	Mc	unknown	Lymphoma	II

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76	Canine	Golden retrievers	M	14	Sarcoma	
77	Canine	Mixed	F	8	Mast cell tumor	
78	Canine	Welsh Corgi	M	10	Cutaneous lymphoma	V
79	Canine	Mixed	F	13	Oral melanoma	II
80	Canine	Mixed	M	7	Sacral neoplasia	
81	Canine	Chow Chow	F	8	Tongue melanoma	V
82	Canine	Mixed	M	15	Melanoma	
83	Canine	Maltese	Fsp	7.5	B cell lymphoma	
84	Canine	Pug	M	9	Mast cell tumor	
85	Canine	Mixed	F	16	Unknown	
86	Canine	Poodle	F	5	GI lymphoma	
87	Canine	Mixed	F	14	Perianal sarcoma	
88	Canine	Beagle	M	12	B cell lymphoma	
89	Canine	Golden retrievers	M	13	Right maxillary sarcoma	IV
90	Canine	Maltese	Fsp	Unknown	Mast cell tumor	III
91	Canine	Dachshunds	M	11	Transitional cell carcinoma	II
92	Canine	Chihuahua	M	13	Nasal tumor	
93	Canine	Mixed	F	12	Nasal adenocarcinoma	IV
94	Canine	Mixed	Fsp	14	Lymphoma	V
95	Canine	Mixed	M	14	Right hindlimb lipoma	

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96	Canine	Mixed	F	13	Malignant thyroid gland tumor	IV
97	Canine	Poodle	F	13	Mammary gland tumor	V
98	Canine	English Bulldog	M	9	Heart tumor	
99	Canine	Schnauzer	F	12	Liver tumor	
100	Canine	Mixed	F	14	Unknown	
101	Canine	Chihuahua	M	7	Histiocytic sarcoma	
102	Canine	Mixed	F	Unknown	Hepatocellular carcinoma	I
103	Canine	Mixed	M	11	Mast cell tumor	II
104	Canine	Golden retrievers	M	13	Nostril mass	
105	Canine	Schnauzer	F	4	Lymphoma	
106	Canine	Husky	F	12	Lymphoma	IV
107	Canine	Border Collies	F	10	Lymphoma	
108	Canine	Shiba Inu	M	8	Mast cell tumor	II
109	Canine	Golden retrievers	M	12	Renal tumor	IV
110	Canine	Husky	M	8	Trichoblastoma	III
111	Canine	Golden retrievers	F	12	T cell lymphoma	
112	Canine	Beagle	M	12	Lymphoma	
113	Canine	Mixed	M	7	B cell lymphoma	IV
114	Canine	Husky	F	5	Lymphoma	V
115	Canine	Schnauzer	F	11	Lymphoma	V

doi:10.6342/NTU202302143

116	Canine	Mixed	F	12	Lymphoma	
117	Canine	Schnauzer	M	13	Prostatic adenocarcinoma	
118	Canine	Shiba Inu	F	8	Lymphoma	
119	Canine	Spitz	M	9	Seminoma	
120	Canine	Schnauzer	Mc	14	Mast cell tumor	I
121	Canine	Mixed	F	11	Lymphoma	IV
122	Canine	Poodle	M	12	Liver tumor	
123	Canine	Dachshunds	F	11	Oral squamous cell carcinoma	II
124	Canine	Poodle	F	8	Mammary gland tumor	
125	Canine	Poodle	M	13	Squamous cell carcinoma, Melanoma	IV
126	Canine	Golden retrievers	F	Unknown	B cell lymphoma	IV
127	Canine	Schnauzer	F	12	Lymphoid nodular hyperplasia	
128	Canine	Mixed	M	9.5	MCT	II
129	Canine	Golden retrievers	F	Unknown	Liver tumor	
130	Canine	Shih Tzu	M	8	Mast cell tumor	II
131	Canine	Mixed	F	13	Chondrosarcoma	I
132	Canine	Mixed	F	11	Mast cell tumor	II
133	Canine	Golden retrievers	Fsp	10	B cell lymphoma	IV
134	Canine	Shiba Inu	M	9	Osteosarcoma	
135	Canine	Mixed	F	13	Mammary carcinoma	II

doi:10.6342/NTU202302143

136	Canine	Beagle	F	9	Transitional cell carcinoma	II
137	Canine	Dachshunds	F	5	B cell lymphoma	
138	Canine	Dachshunds	Fsp	6	Hepatomegaly, IVD	
139	Canine	Mixed	F	10	Mast cell tumor	I
140	Canine	Golden retrievers	Mc	10	Mast cell tumor	II
141	Canine	Mixed	Fsp	13	Malignant ameloblastoma	
142	Canine	Schnauzer	M	12	Left nasal carcinoma	II
143	Canine	Maltese	F	9	Lymphoma	IV
144	Canine	Mixed	M	11	right adrenal gland tumor and pancreatic tumor	
145	Canine	Poodle	M	3	Spleen tumor	
146	Canine	Welsh Corgi	M	4	B cell lymphoma	IV
147	Canine	Maltese	M	11	Transitional cell carcinoma	II
148	Canine	Mixed	F	Unknown	Mast cell tumor	III
149	Canine	Schnauzer	F	9.5	Trigeminal nerve sheath tumor	
150	Canine	Golden retrievers	Fsp	8	Adenocarcinoma	
151	Canine	Welsh Corgi	M	6.5	B cell lymphoma	IV
152	Canine	Golden retrievers	F	8	Unknown	II
153	Canine	Poodle	M	12	Nasal neoplasia	
154	Canine	Mixed	M	9.8	Mammary gland tumor	II
155	Canine	Husky	M	11	Unknown	

doi:10.6342/NTU202302143

156	Canine	Mixed	F	14	Unknown	IV
157	Canine	Mixed	F	11	Oral sarcoma	
158	Canine	Shih Tzu	M	11	mass of the right hindlimb	
159	Canine	French Bulldog	M	8	Lymphoma	
160	Canine	Yorkshire	F	10	Unknown	IV
161	Canine	Schnauzer	Mc	12	Liver tumor	
162	Canine	Golden retrievers	Fsp	7	Lymphoma	II
163	Canine	Maltese	M	12	Unknown	
164	Canine	Mixed	Fsp	10	Mast cell tumor	II
165	Canine	Mixed	F	10	digit mass	
166	Canine	Mixed	M	9	Rectum mass	
167	Canine	Labrador	M	9	Splenic hemangiosarcoma	III
168	Canine	Golden retrievers	M	Unknown	Liver mass	
169	Canine	Mixed	F	Unknown	Unknown	V
170	Canine	Poodle	M	10	Gastric mass	
171	Canine	Maltese	F	7.5	Mast cell tumor	III
172	Canine	Beagle	M	12	Liver mass	
173	Canine	Chihuahua	Fsp	11	Unknown	V
174	Canine	Schnauzer	F	12	Mammary gland tumor	III
175	Canine	Mixed	M	11	L7 giant cell tumor	

doi:10.6342/NTU202302143

176	Canine	Mixed	F	9	Rt scapular mass	
177	Canine	Beagle	M	13	Abdominal seroma and splenic mass	
178	Canine	Shiba Inu	M	11	Splenic hematoma	
179	Canine	Golden retrievers	F	15	Liver and spleen masses	
180	Canine	Poodle	F	10	Unknown	II
181	Canine	Standard Poodle	F	9	Oral melanoma	V
182	Canine	Beagle	Fsp	10	Pheochromocytoma	V
183	Canine	Dachshunds	M	1.5	Soft tissue sarcomas	V
184	Canine	Mixed	F	7	Perianal mass	IV
185	Canine	Maltese	F	7	Lymphoma	V
186	Canine	Poodle	M	8	Mast cell tumor	II
187	Canine	Mixed	F	10	Mast cell tumor	III
188	Canine	Beagle	Mc	11	Sarcoma	V
189	Canine	Schnauzer	F	5	Hepatic cholecystitis	
190	Canine	Mixed	M	13	Oral PNST (peripheral nerve sheath tumor)	I
191	Canine	Golden retrievers	Mc	11	Lymphoma	IV
192	Canine	Golden retrievers	F	11	Lymphoma	III
193	Canine	Terrier	Fsp	8	Lymphoma	III
194	Canine	Mixed	F	10	from right abdomen mass	
195	Canine	Mixed	M	8	Granuloma from thoracic dorsal line mass	

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196	Canine	Mixed	F	8	Intestinal adenocarcinoma	
197	Canine	Mixed	F	8	Splenomegaly	
198	Canine	Pit Bull	F	6	Popliteal lymph node enlargement	
199	Canine	Welsh Corgi	M	5	Primary lung tumor	
200	Canine	Poodle	F	11	B cell lymphoma	V
201	Canine	Schnauzer	M	13	Lymphoma	
202	Canine	cocker spaniel	F	Unknown	Transitional cell carcinoma	IV
203	Canine	Mixed	Fsp	10	Unknown	I
204	Canine	Mixed	Mc	Unknown	Fibrosarcoma	
205	Canine	Mixed	F	9	Unknown	
206	Canine	Terrier	Fsp	Unknown	B cell lymphoma	IV
207	Canine	Poodle	M	9	Lipoma	
208	Canine	Mixed	F	7	Bilateral mass	
209	Canine	Dachshunds	F	12	Sinonasal adenocarcinoma	III
210	Canine	Maltese	F	10	Anemia	
211	Canine	Mixed	F	14	Liver mass: Cholangiohepatitis	
212	Canine	Mixed	M	13	Oral melanoma	V
213	Canine	Mixed	Fsp	8	Right lower lip melanoma	
214	Canine	Poodle	Mc	11	Ectopic thyroid tumor	
215	Canine	French Bulldog	F	8	Pulmonary neuroendocrine tumor	

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216	Canine	Beagle	Fsp	Unknown	Cranial mediastinal tumor	
217	Canine	Shiba Inu	Mc	4	Fibrosarcoma	II
218	Canine	French Bulldog	M	11	Osteosarcoma	
219	Canine	Poodle	F	Unknown	pancreas endocrine carcinoma	
220	Canine	Mixed	M	10	Spleen and liver mass	
221	Canine	Mixed	F	13	Fibrosarcoma	III
222	Canine	Maltese	F	9	Unknown	
223	Canine	Schnauzer	M	12	Caudal hock apocrine adenocarcinoma	II
224	Canine	Mixed	Mc	10	Lymphoma	V
225	Canine	Maltese	M	10	one small nodule 0.7-0.8 cm at 2nd left MG	
226	Canine	Schnauzer	M	12	Dorsal neck mass	
227	Canine	Mixed	F	11	B cell lymphoma	IV
228	Canine	Beagle	Mc	10	Mast cell tumor	
229	Canine	Golden retrievers	M	12	Lung mass, renal tumor	
230	Canine	Maltese	M	12	B cell lymphoma	V
231	Canine	Golden retrievers	F	13	Thyroid gland adenocarcinoma	
232	Canine	Mixed	F	11	Vaginal leiomyosarcoma	
233	Canine	Golden retrievers	F	Unknown	Unknown	IV
234	Canine	Poodle	F	8.8	Adenocarcinoma	IV
235	Canine	Poodle	F	11	Mast cell tumor	

doi:10.6342/NTU202302143

236	Canine	Mixed	F	6	CLL	IV
237	Canine	Golden retrievers	F	10	Lymphoma	III
238	Canine	Poodle	M	9	Malignant melanoma	III
239	Canine	Mixed	M	10	Sebaceous carcinoma	
240	Canine	Mixed	M	12	Iridal mass	
241	Canine	Terrier	Fsp	12	Adrenal gland tumor	
242	Canine	Shiba Inu	M	13	Histiocytic sarcoma	
243	Canine	Golden retrievers	F	13	Multicentric lymphoma	V
244	Canine	bear dog	F	9	Thyroid gland tumor	III
245	Canine	Mixed	F	8	Mast cell tumor	
246	Canine	Pomeranian	M	Unknown	Nasal tumor	
247	Canine	Mixed	F	Unknown	Mammary gland tumor	I
248	Canine	Poodle	M	Unknown	Malignant melanoma	III
249	Canine	French Bulldog	M	11	Apocrine ductal carcinoma	I
250	Canine	Dachshunds	M	10	B cell lymphoma	III
251	Canine	Golden retrievers	M	10	Insulinoma	
252	Canine	Maltese	M	9.8	Urine Bladder mass	
253	Canine	Labrador	F	11	Hemangiosarcoma	
254	Canine	Pomeranian	F	Unknown	Transitional cell carcinoma	II
255	Canine	Poodle	F	Unknown	Mast cell tumor	I

doi:10.6342/NTU202302143

256	Canine	Schnauzer	M	15	Left anal sac apocrine adenocarcinoma	II
257	Canine	Border Collies	M	2	Adenocarcinoma	V
258	Canine	Yorkshire	F	4.5	Multicentric lymphoma	IV
259	Canine	French Bulldog	M	14	Squamous cell carcinoma	IV
260	Canine	Mixed	M	10	T cell lymphoma	V
261	Canine	Mixed	M	Unknown	Basal cell carcinoma	III
262	Canine	Mixed	F	10	Transitional cell carcinoma	II
263	Canine	Dachshunds	M	Unknown	Lymphoma	V
264	Canine	Schnauzer	F	Unknown	Facial peripheral nerve sheath tumor	
265	Canine	Cavalier	M	Unknown	Multicentric lymphoma	IV
266	Canine	Shih Tzu	M	8	Left caudal back mass	
267	Canine	Maltese	F	Unknown	GI lymphoma	IV
268	Canine	Schnauzer	F	Unknown	Hemangiosarcoma	I
269	Canine	Poodle	F	Unknown	Pulmonary adenocarcinoma	
270	Canine	French Bulldog	M	Unknown	Adenocarcinoma	III
271	Canine	Mixed	M	Unknown	Lymphoma	III
272	Canine	Wolfhound	F	Unknown	Mast cell tumor	II
273	Canine	Poodle	M	18	Unknown	
274	Canine	Doberman	Fsp	5	B cell lymphoma	
275	Canine	Maltese	M	Unknown	Lymphoma	IV

doi:10.6342/NTU202302143

276	Canine	Mixed	F	Unknown	Mast cell tumor	IV
277	Canine	Mixed	F	Unknown	Histiocytic sarcoma	
278	Canine	Poodle	M	6	Hematemesis	
279	Canine	Mixed	F	Unknown	GI lymphoma	III
280	Canine	Schnauzer	M	Unknown	Fibrosarcoma	I
281	Canine	Husky	M	Unknown	Biliary origin tumor	II
282	Canine	Dachshunds	M	Unknown	B cell lymphoma	V
283	Canine	Spitz	M	11	Unknown	II
284	Canine	Golden retrievers	F	8	Sarcoma	
285	Canine	Spitz	F	11	T cell lymphoma	IV
286	Canine	Airedale Terrier	Fsp	13	Transitional cell carcinoma	IV
287	Canine	Dachshunds	F	9	Non-functional left thyroid carcinoma	
288	Canine	Terrier	F	0.9	PSS	

doi:10.6342/NTU202302143

Table 2. DR-70, KMO, and Ki-67concentrationsn in different types of cancer and stages

	DR-70 (μg/mL)			KMO (ng/mL)			Ki-67 (ng/mL)		
	Con.	**Number (%)**	***P*-value**	**Con.**	**Number (%)**	***P*-value**	**Con.**	**Number (%)**	***P*-value**
Healthy	2.18 ± 0.61	68 (31.34 %)	<0.0001*	2.95 ± 3.63	34 (31.48 %)	<0.0001*	4.14 ± 0.92	32 (33.33 %)	0.892
Tumor type*									
Lymphoma	2.18 ± 0.61	68 (31.34 %)		2.95 ± 3.63	34 (31.48 %)		4.14 ± 0.92	32 (33.33 %)	
Mast cell tumor	2.04 ± 0.57	28 (12.9 %)		3.14 ± 2.94	18 (16.67 %)		4.12 ± 0.87	17 (17.71 %)	
Sarcoma	2.26 ± 0.58	28 (12.90 %)		3.71 ± 5.01	12 (11.11 %)		4.08 ± 1.43	11 (11.46 %)	
Carcinoma	2.17 ± 0.68	27 (12.44 %)		2.52 ± 2.22	11 (10.19 %)		3.91 ± 0.91	8 (8.33 %)	
Melanoma	2.14 ± 0.53	17 (7.83 %)		3.53 ± 2.74	9 (8.33 %)		4.21 ± 1.41	8 (8.33 %)	

doi:10.6342/NTU202302143

Mammary gland tumor	1.92 ± 0.67	15 (6.91 %)		4.41 ± 5.59	11 (10.19 %)		4.22 ± 1.14	9 (9.38 %)	
Adenocarcinoma	2.16 ± 0.78	15 (6.91 %)		2.27 ± 0.94	5 (4.63 %)		3.67 ± 1.41	4 (4.17 %)	
Transitional cell carcinoma	2.19 ± 0.80	13 (5.99 %)		2.14 ± 1.71	4 (3.70 %)		3.75 ± 0.73	3 (3.13 %)	
Squamous cell carcinoma	2.26 ± 0.55	6 (2.76 %)		2.11 ± 0.94	4 (3.70 %)		4.15 ± 1.20	4 (4.17 %)	
Stage#									
I	2.04 ± 0.62	16 (11.11 %)	0.1391	3.29 ± 2.36	8 (14.29 %)	0.9023	4.37 ± 0.91	8 (14.81 %)	0.6636
II	2.04 ± 0.60	34 (23.61 %)		3.71 ± 4.75	19 (33.93 %)		3.76 ± 1.23	16 (29.63 %)	
III	2.24 ± 0.74	28 (19.44 %)		2.29 ± 1.18	9 (16.07 %)		3.90 ± 1.27	9 (16.67 %)	

doi:10.6342/NTU202302143

IV &V	2.29 ± 0.60	66 (45.83 %)		2.30 ± 1.92	20 (35.71 %)	4.09 ± 0.92	21 (38.89 %)

*Unpaired t test; #one ANOVA test; $P < 0.05$ show significant difference; $P < 0.01$ show extremely significant difference.

Con.= Concentration

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Table 3. The concentration of DR-70, KMO and Ki-67 in gender, age, and breed

	DR-70 (μg/mL)			KMO (ng/mL)			Ki-67 (ng/mL)		
	Con.	**Number (%)**	***P*-value**	**Con.**	**Number (%)**	***P*-value**	**Con.**	**Number (%)**	***P*-value**
Gender									
Female	2.16 ± 0.70	155 (54.01 %)	0.6859	2.87 ± 3.14	70 (51.47 %)	0.4236	4.16 ± 1.28	60 (51.28 %)	0.2982
Male	2.12 ± 0.66	133 (46.18 %)		2.71 ± 3.33	66 (48.53 %)		4.47 ± 1.52	57 (48.72 %)	
Age									
< 10 years old	2.04 ± 0.68	107 (43.32 %)	0.0532	3.36 ± 4.36	50 (36.68 %)	0.5030	4.20 ± 1.26	46 (39.32 %)	0.2626
≧ 10 years old	2.17 ± 0.68	140 (56.68 %)		2.39 ± 2.28	76 (60.32 %)		4.47 ± 1.54	62 (52.99 %)	
Breed									
Mixed	2.22 ± 0.65	88 (30.45 %)	0.0626	2.63 ± 2.94	43 (32.58 %)	0.5791	4.49 ± 1.26	37 (31.62 %)	0.3439
Poodle	2.20 ± 0.73	29 (10.03 %)		3.67 ± 3.86	12 (9.09 %)		4.62 ± 0.95	13 (11.11 %)	

doi:10.6342/NTU202302143

Schnauzer	1.95 ± 0.48	27 (9.34 %)	2.39 ± 1.54	12 (9.09 %)	4.73 ± 2.45	15 (12.82 %)
Golden retriever	2.08 ± 0.48	25 (8.65 %)	6.21 ± 7.19	11 (8.33 %)	3.84 ± 1.74	11 (9.40 %)
Maltese	2.27 ± 0.54	18 (6.23 %)	1.62 ± 1.30	6 (4.55 %)	4.91 ± 1.01	3 (2.56 %)
Dachshund	2.16 ± 0.85	14 (4.84 %)	1.76 ± 3.52	6 (4.55 %)	3.52 ± 0.63	6 (5.13 %)
Beagle	2.02 ± 1.44	11 (3.11 %)	1.44 ± 2.18	5 (3.79 %)	4.42 ± 1.17	2 (1.71 %)
Shiba Inu	1.68 ± 0.58	8 (2.77 %)	2.78 ± 1.86	5 (3.79 %)	3.97 ± 0.17	3 (2.56 %)
French Bulldog	2.41 ± 1.26	9 (3.11 %)	1.93 ± 1.17	2 (1.52 %)	3.33 ± 1.01	2 (1.71 %)
Welsh Corgi	2.66 ± 0.81	6 (2.08 %)	1.96 ± 1.27	4 (3.03 %)	4.23 ± 0.81	4 (3.42 %)
Husky	2.13 ± 0.36	6 (2.08 %)	1.87 ± 1.63	5 (3.79 %)	4.27 ± 1.30	5 (4.27 %)
Border Collies	1.64 ± 0.38	9 (3.11 %)	4.07 ± 3.47	5 (3.79 %)	4.33 ± 1.11	4 (3.42 %)

*Unpaired t test; #one ANOVA test; $P < 0.05$ show significant difference; $P < 0.01$ show extremely significant difference.

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Table 4 Sensitivity and specificity of single or combined detections of plasma DR-70, KMO, and Ki-67 levels in tumor dogs

Tumor marker	AUC	Sensitivity (%)	Specificity (%)	Cutoff
DR-70	0.898	82.8	79.4	2.0
KMO	0.809	82.8	65.6	1.9
Ki-67	0.553	67.2	46.1	2.3
DR-70+ KMO	0.889	72.4	90.6	3.1
DR-70+ Ki-67	0.932	94.8	80	1.8
KMO+ Ki-67	0.834	98.3	61.1	1.7
DR-70+ KMO+ Ki-67	0.934	100	74.4	1.3

doi:10.6342/NTU202302143

Figures

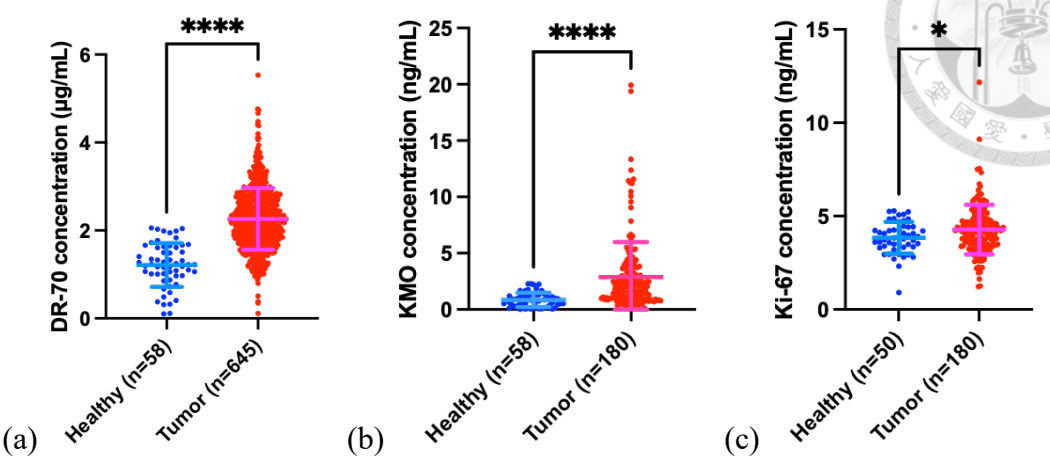

Figure 1. The levels of DR-70, KMO, and Ki-67 expression in plasma are considerably higher in tumor dogs compared to healthy dogs.

The levels of (a) DR-70, (b) KMO, and (c) Ki-67 expression was found to be significantly different (DR-70 & KMO, $P< 0.0001$; Ki-67, $P< 0.05$) between the healthy and tumor dog groups.

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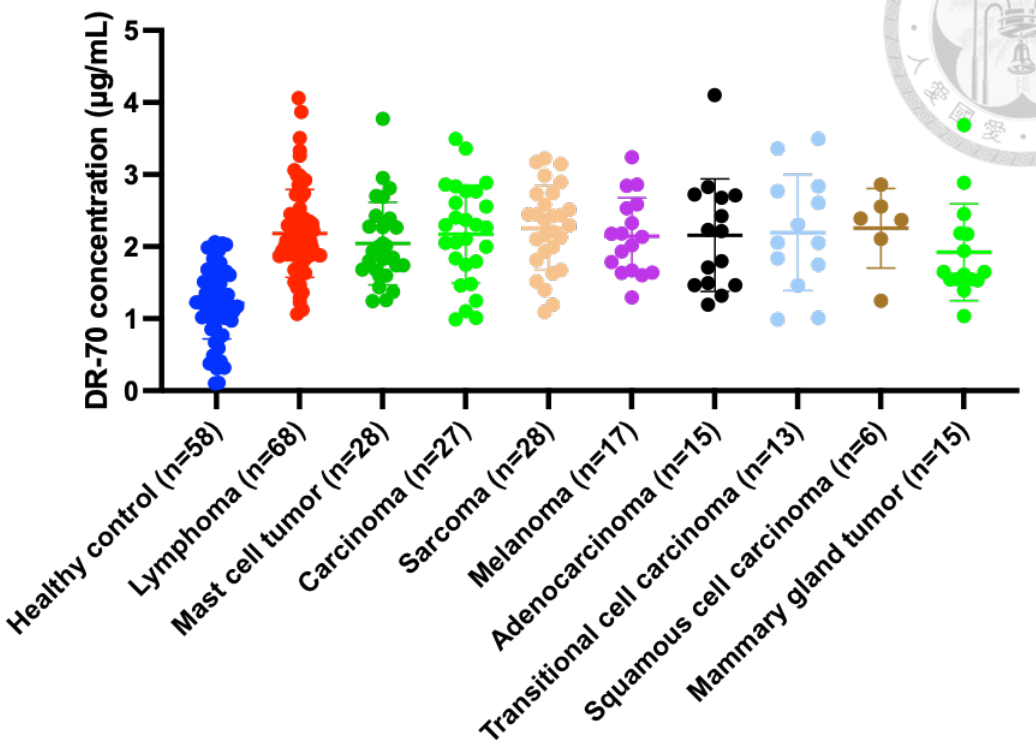
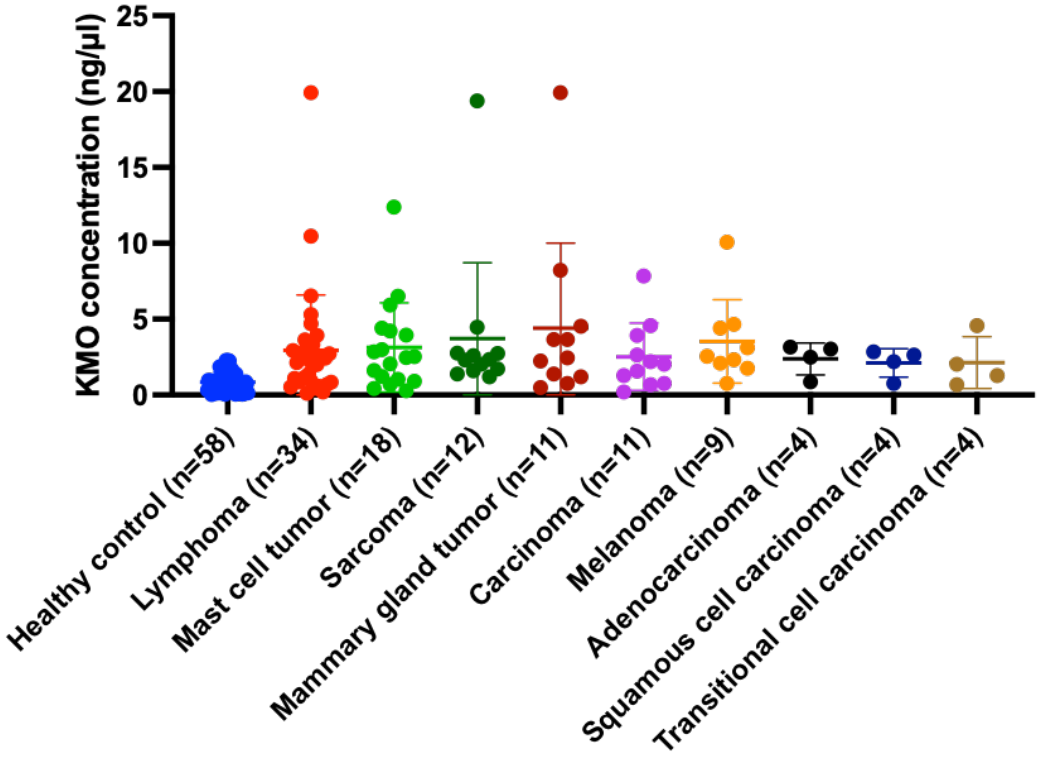
(a)



(b)



(c)

doi:10.6342/NTU202302143

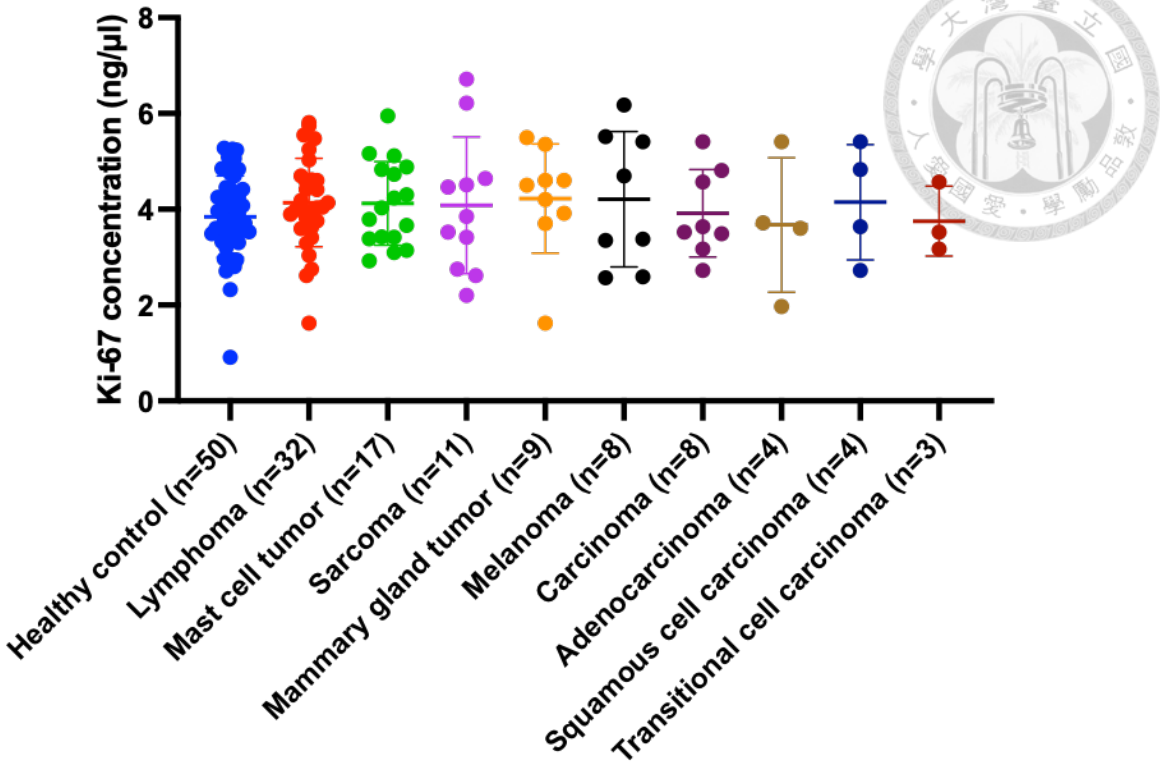


Figure 2. Overexpression of DR-70, KMO, and Ki-67 in different tumor types.

The levels of (a) DR-70 and (b) KMO were higher in different types of tumor dogs compared to healthy ones (P<0.0001). (c) Ki-67 expression in different types of tumor dogs (P=0.892).

doi:10.6342/NTU202302143

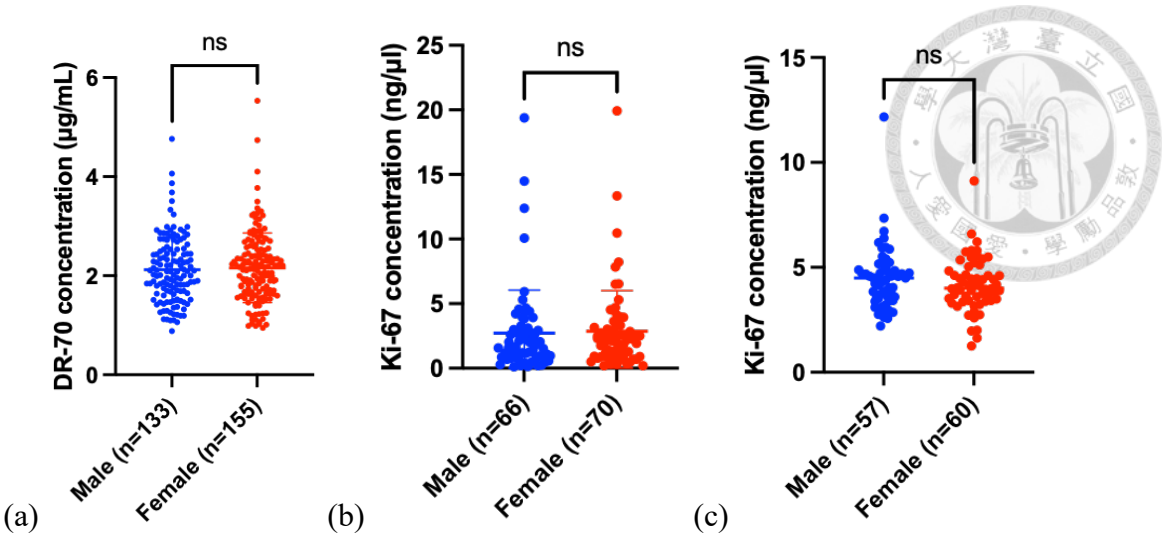


Figure 3. Gender differences in the expression of DR-70, KMO and Ki-67

There was no statistically significant difference in (a) DR-70 (*P*=0.6859), (b) KMO (*P*=0.4236), and (c) Ki-67 (*P*=0.2982) expression levels between males and females.

doi:10.6342/NTU202302143

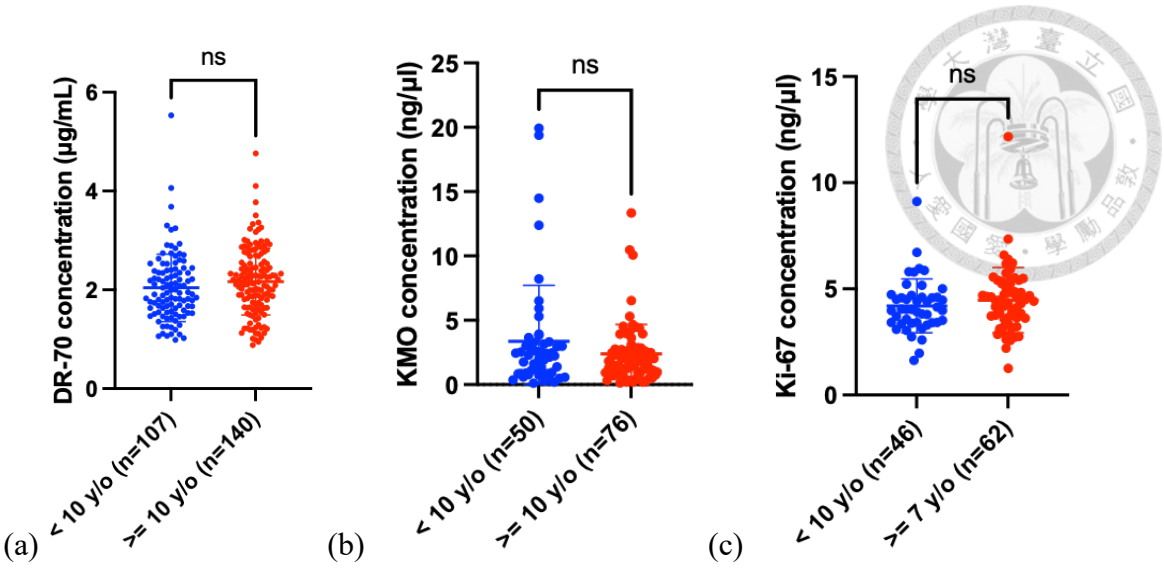
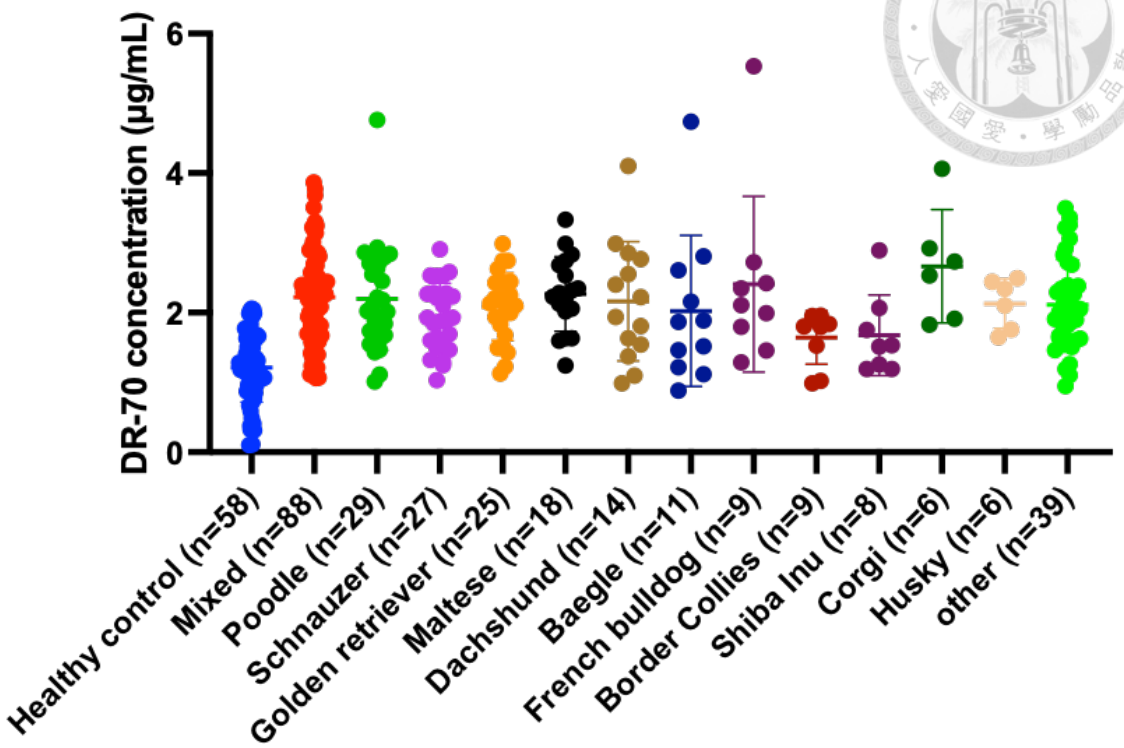


Figure 4. Expression levels of DR-70, KMO, and Ki-67 in different age groups.

There was no statistically significant difference in (a) DR-70 (*P*=0.0532), (b) KMO (*P*=0.5030), and (c) Ki-67 (*P*=0.2626) expression levels between the <10 y/o and >=10 y/o age groups.

doi:10.6342/NTU202302143

(a)



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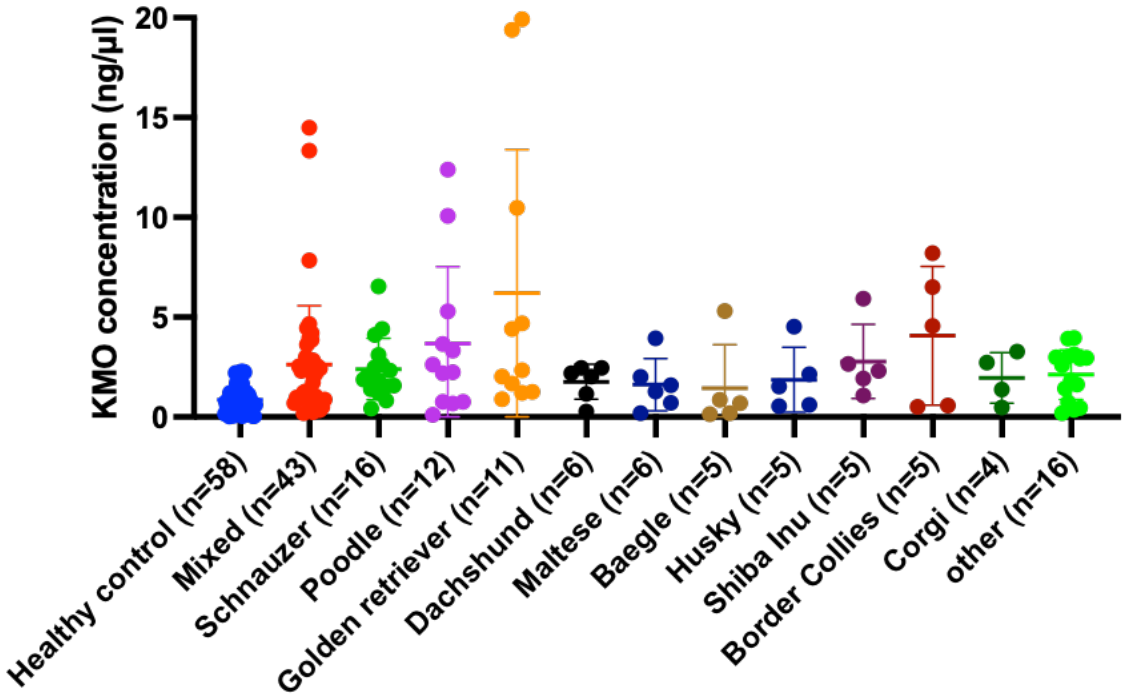

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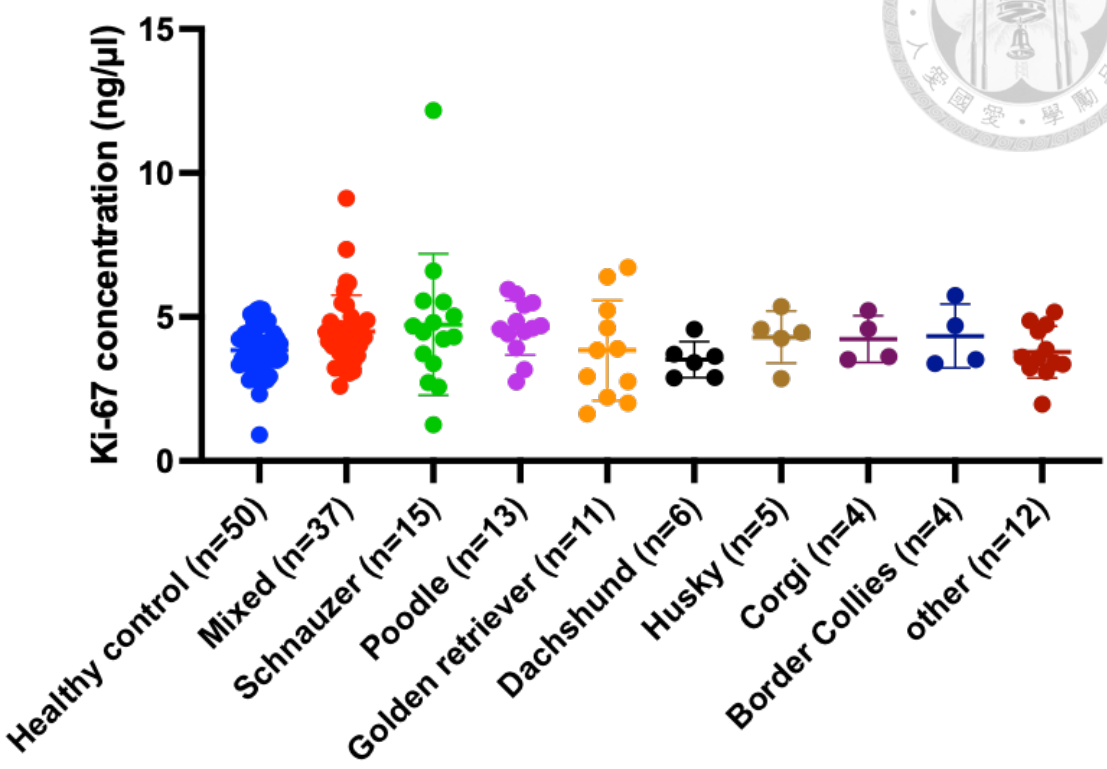
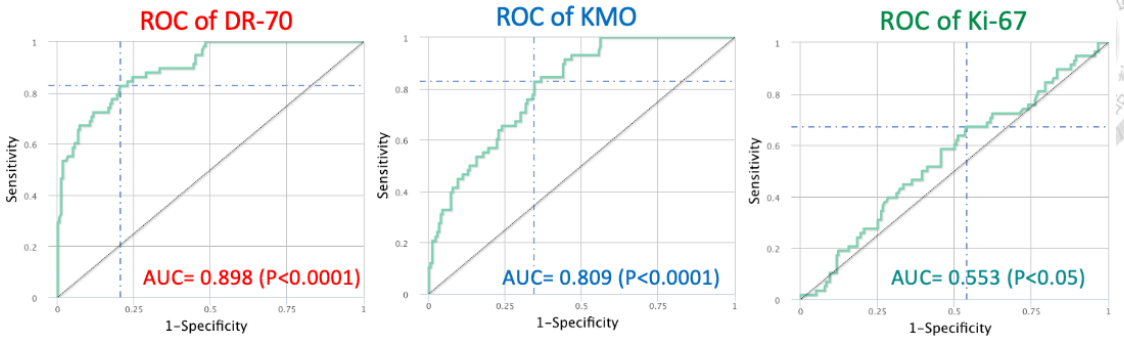


Figure 5. Expression levels of DR-70, KMO, and Ki-67 in different dog breeds.

There was no statistically significant difference in (a) DR-70 (*P*=0.0626), (b) KMO (*P*=0.5791), and (c) Ki-67 (*P*=0.3439) expression levels among the dog breeds.

doi:10.6342/NTU202302143

(a)



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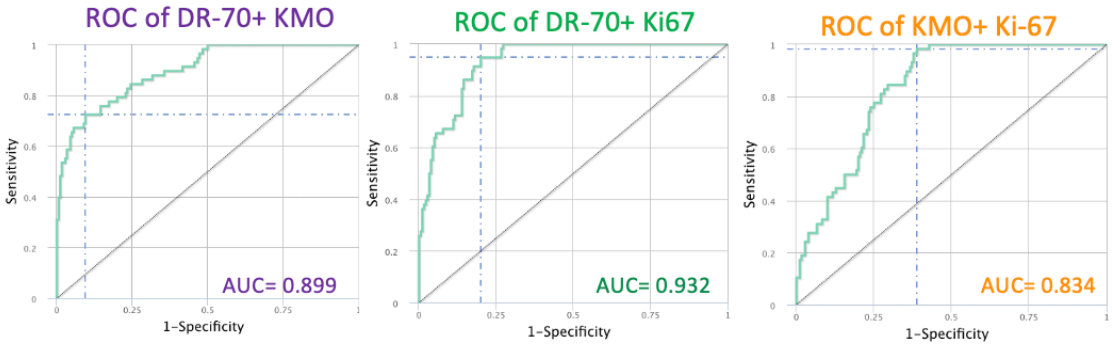

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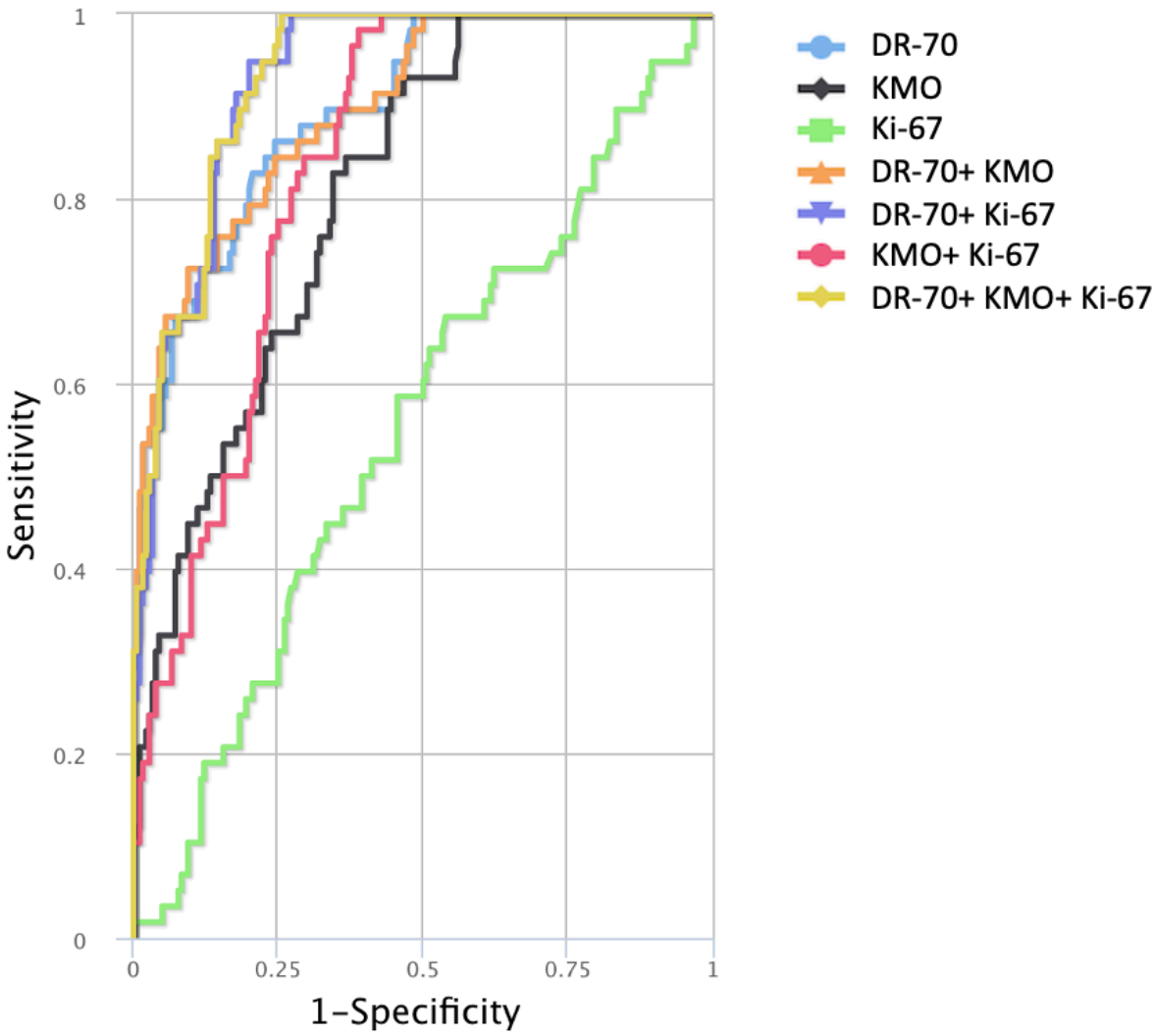
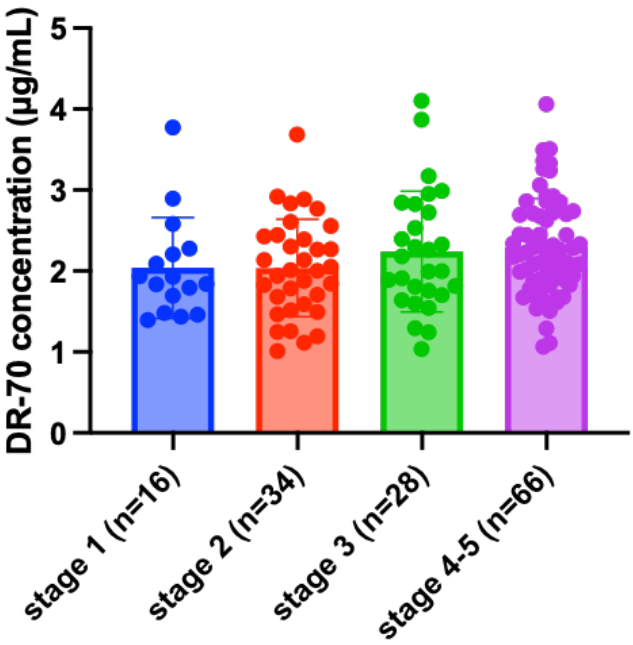


Figure 6. The ROC curve of single and multiple biomarkers combined in canine cancer diagnosis.

(a) ROC curve of single marker detection of DR-70, KMO, and Ki-67**.** (b) ROC curve of double markers detection of DR-70+ KMO, DR-70+ Ki-67, KMO+ Ki-67. (c) ROC curve of three cancer biomarkers combined detection of DR-70+ KMO+ Ki-67.

doi:10.6342/NTU202302143

(a)



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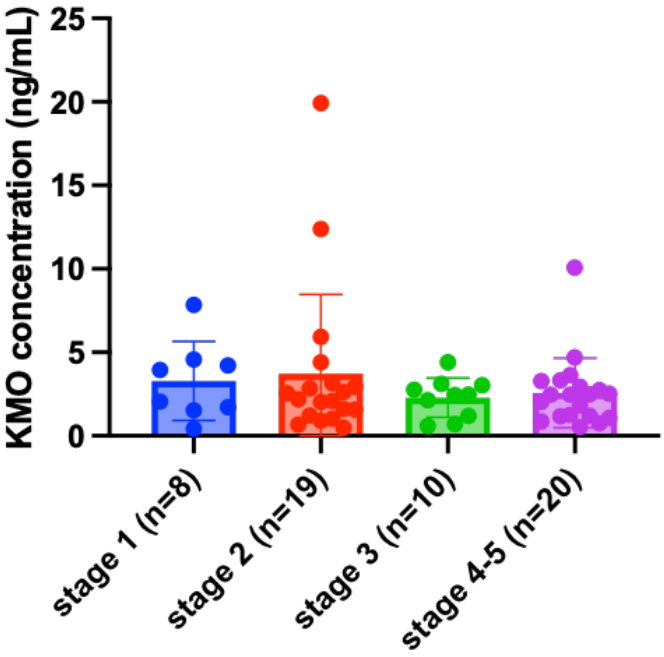

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doi:10.6342/NTU202302143

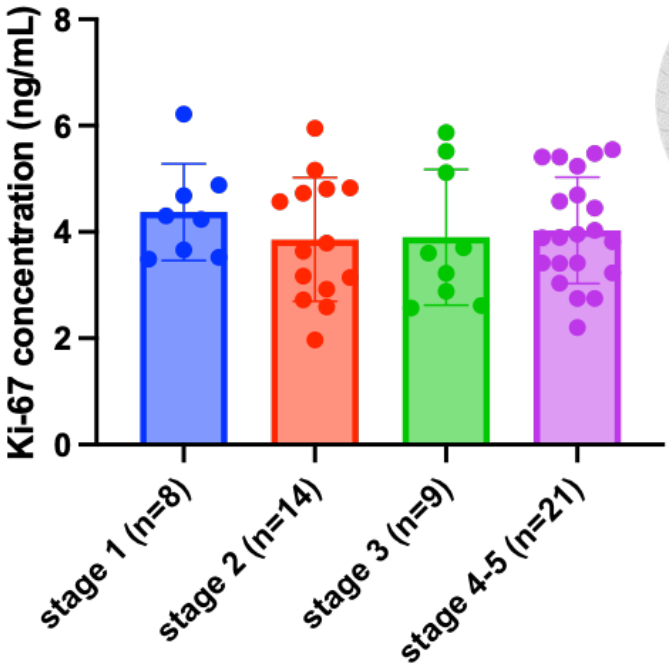
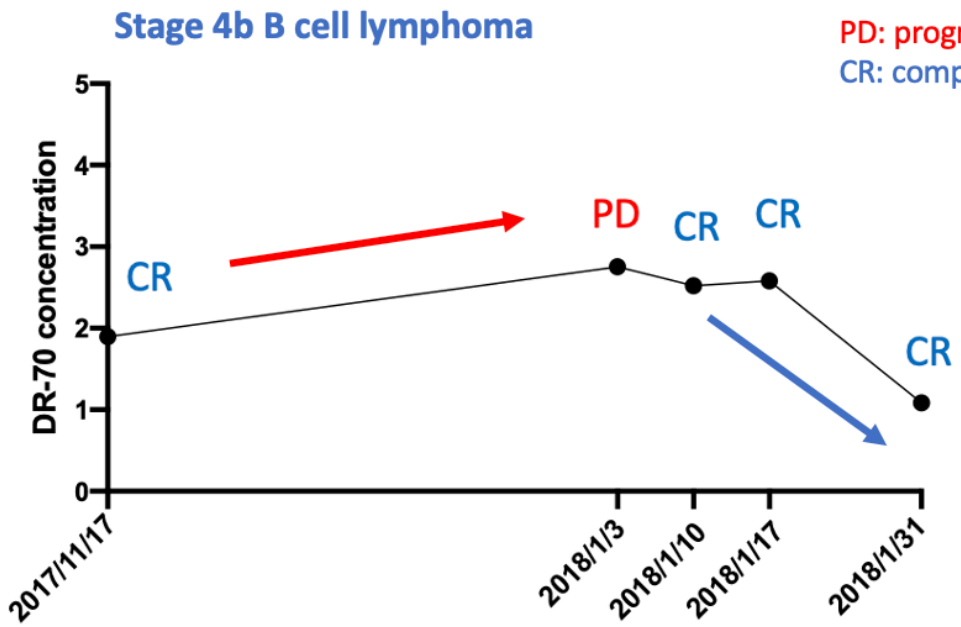


Figure 7 Expression levels of DR-70, KMO, and Ki-67 across different stages of tumors.

There was no statistically significant difference in (a) DR-70 (*P*=0.1391), (b) KMO (*P*=0.9023), and (c) Ki-67 (*P*=0.6636) expression levels among the tumor stages.

doi:10.6342/NTU202302143

(a)



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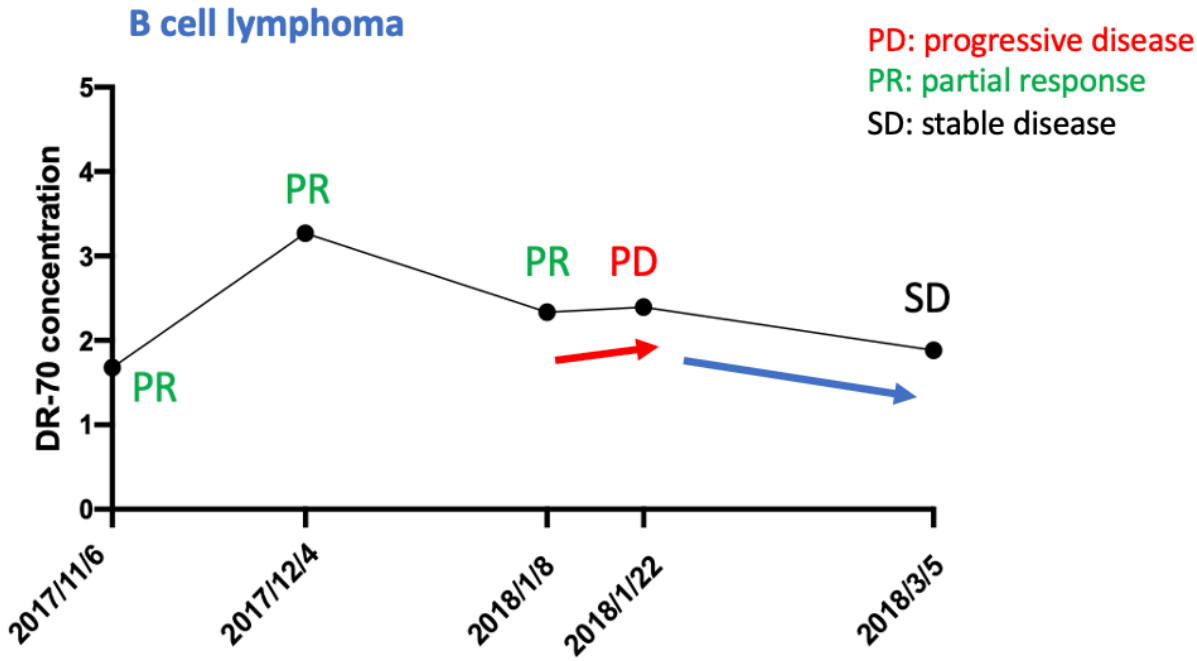

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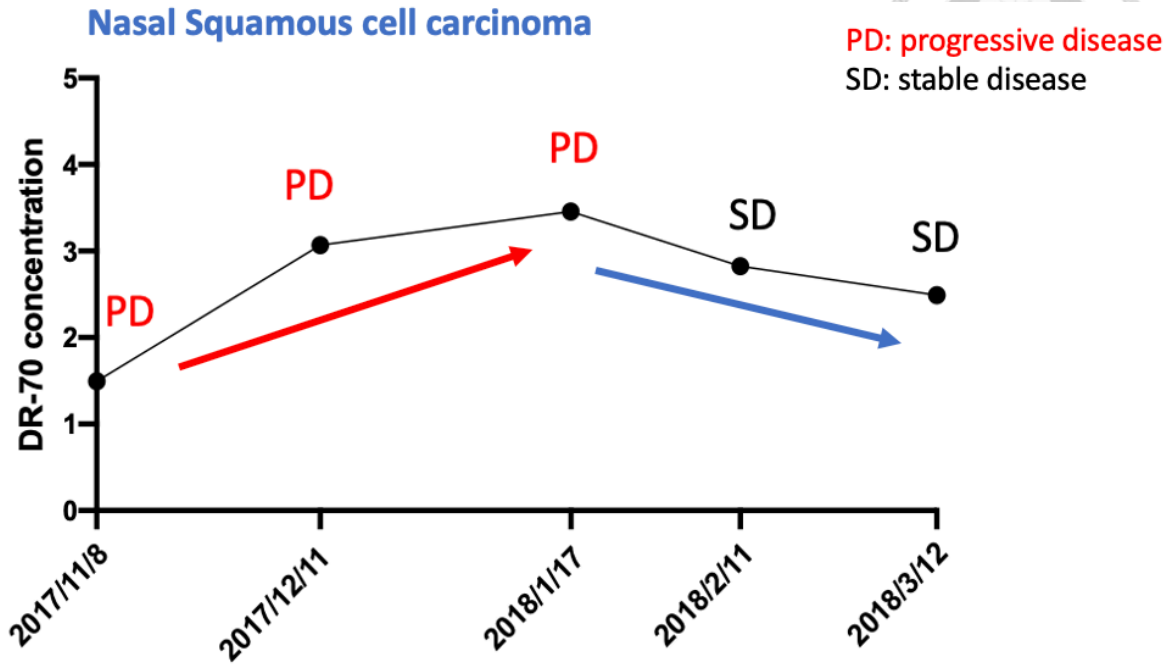

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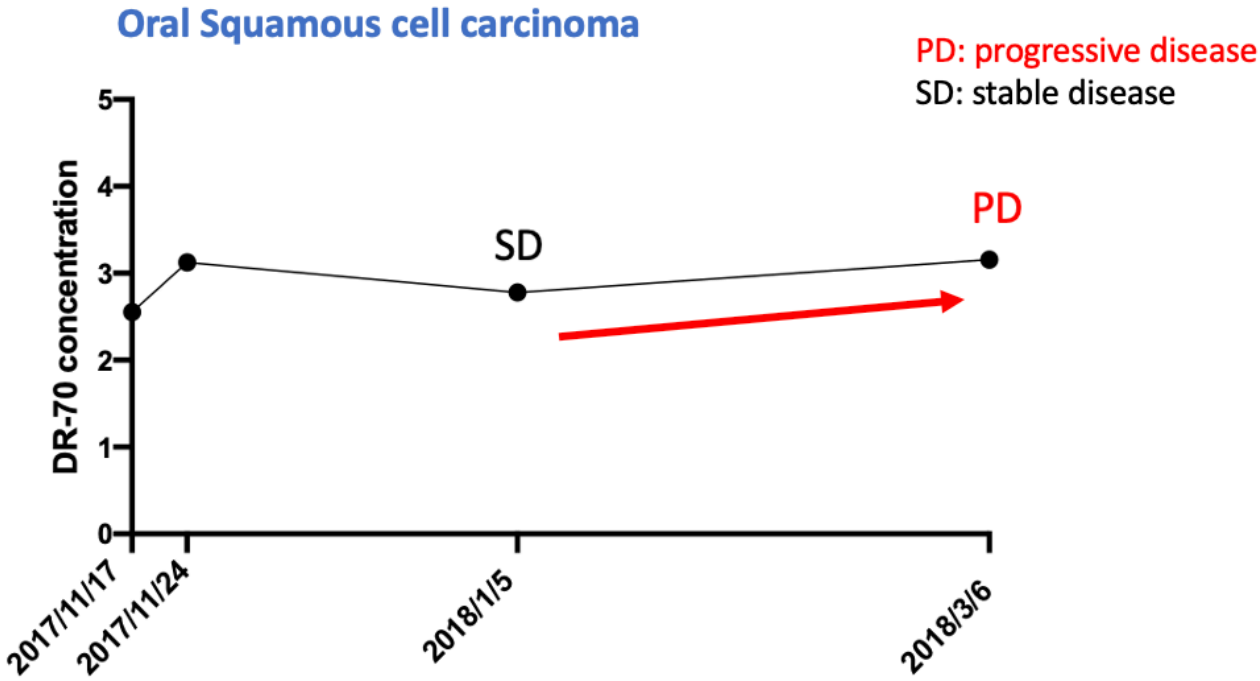

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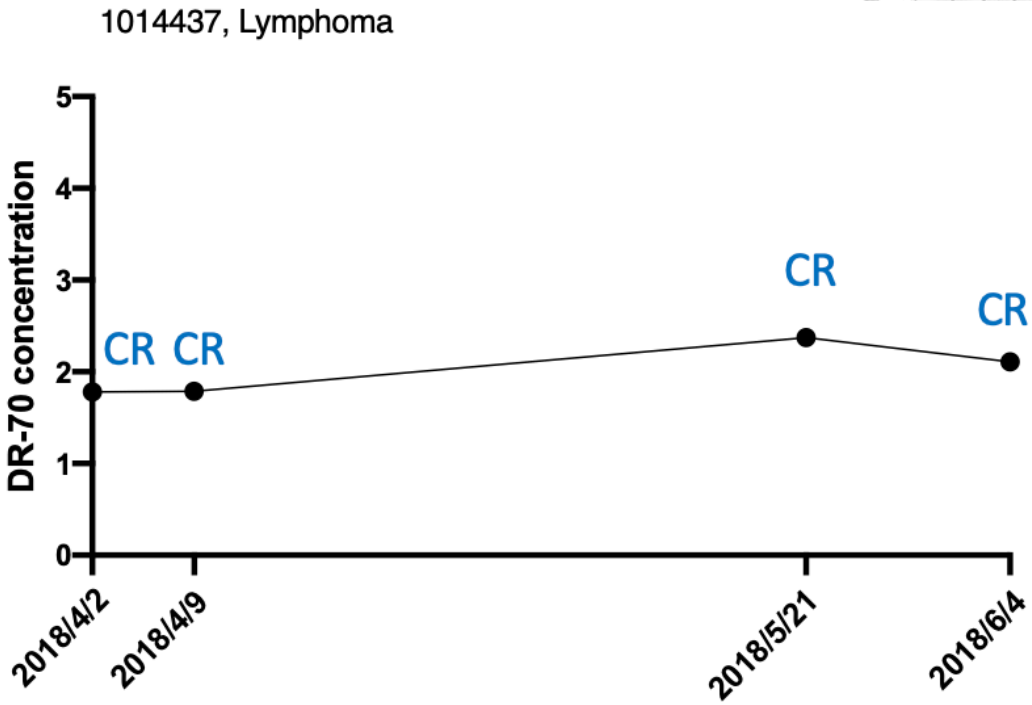
(e)



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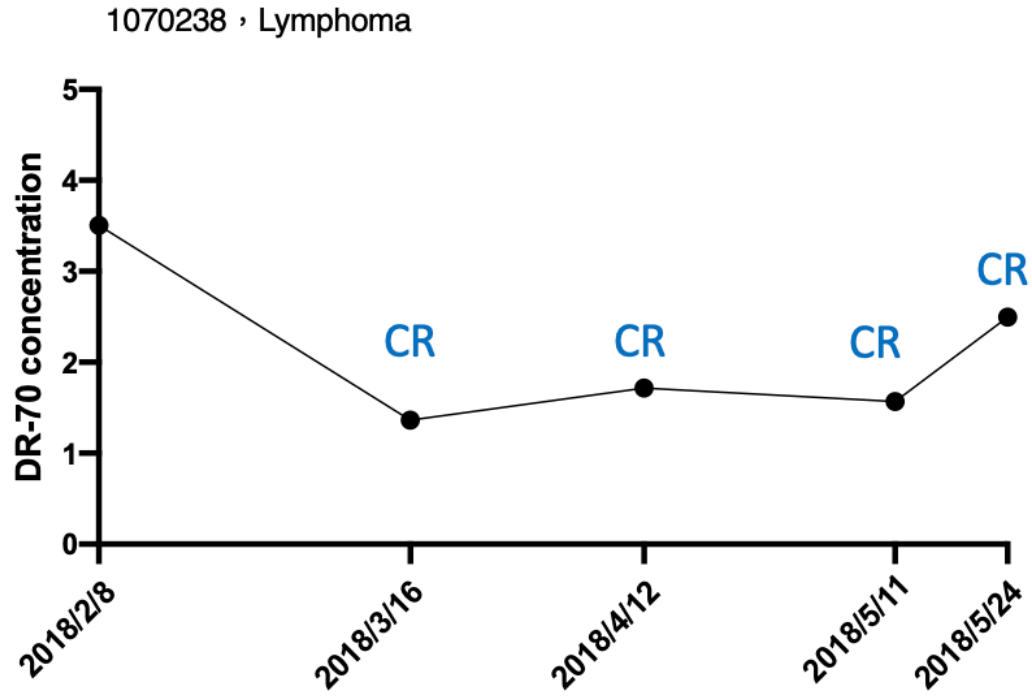

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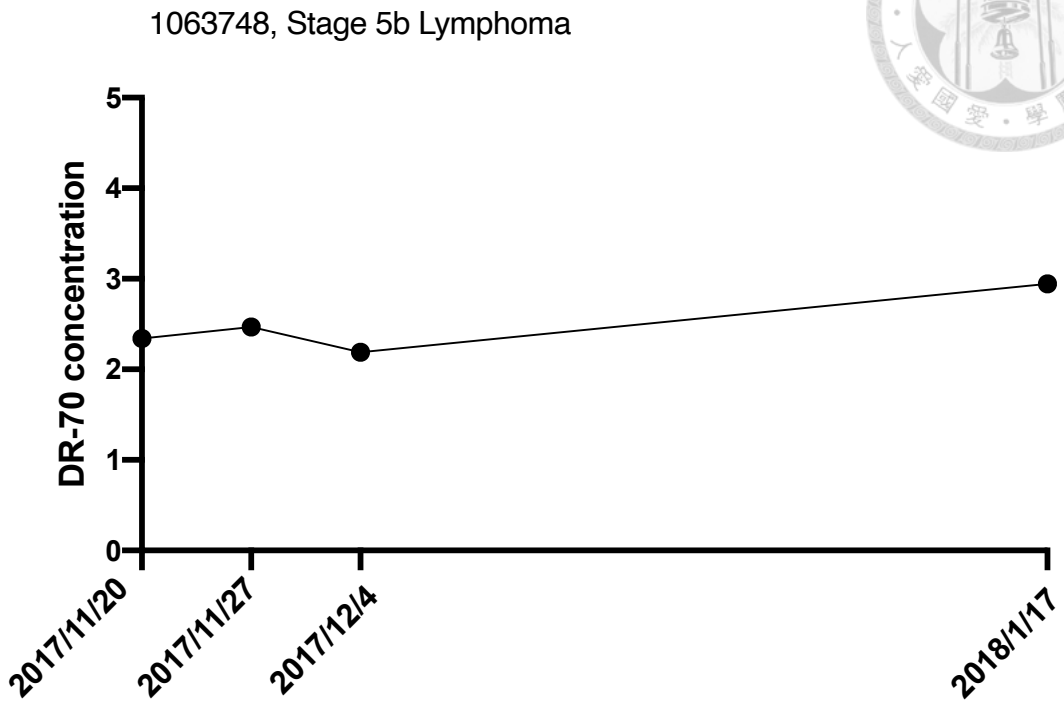
(g)



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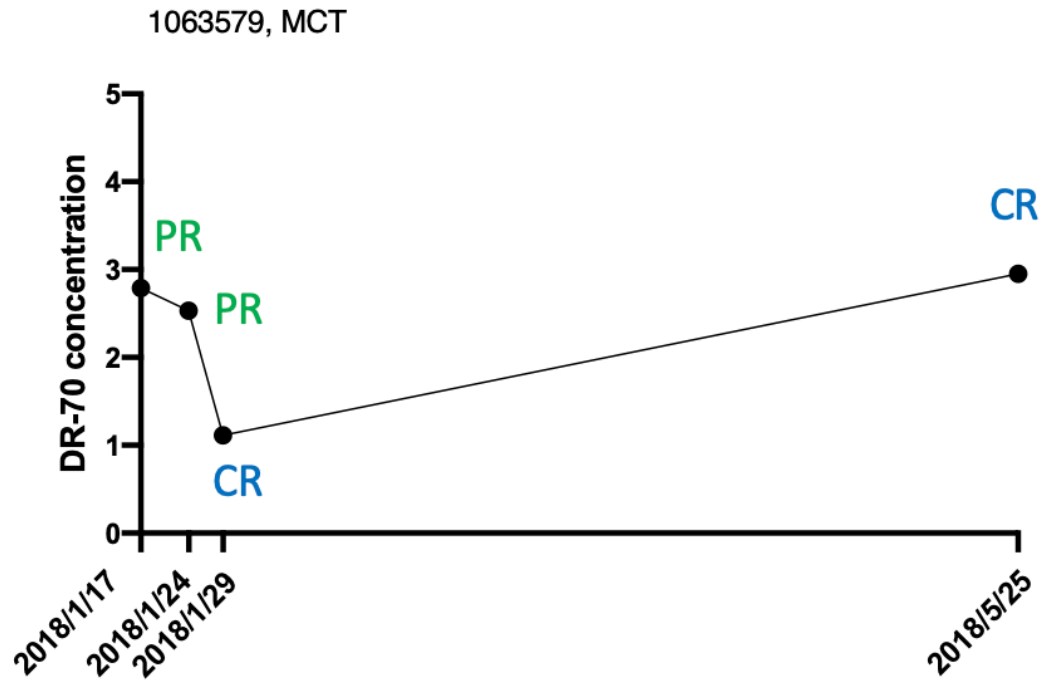

Figure 8. The correlation between tumor progression and plasma DR-70 concentration

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(a) DR-70 levels of stage 4b B cell lymphoma patient increased when the tumor size was diagnosed as progressive disease (PD), and decreased when the tumor size was diagnosed as complete response (CR). (b) The changes in DR-70 level in a B cell lymphoma patient during the treatment of cancer, DR-70 concentration was proportional to tumor burden or volume. (c) In the Nasal squamous cell carcinoma patient, the level of DR-70 increased when the tumor size was diagnosed as PD and decreased when the tumor size was diagnosed as stable disease (SD). (d) In the oral squamous cell carcinoma patients during the cancer process, when the tumor size was diagnosed as PD, DR-70 concentration increased. (e-h) Three lymphoma patients and one mast cell tumor patients had DR-70 expression changes but the tumor size was maintained.

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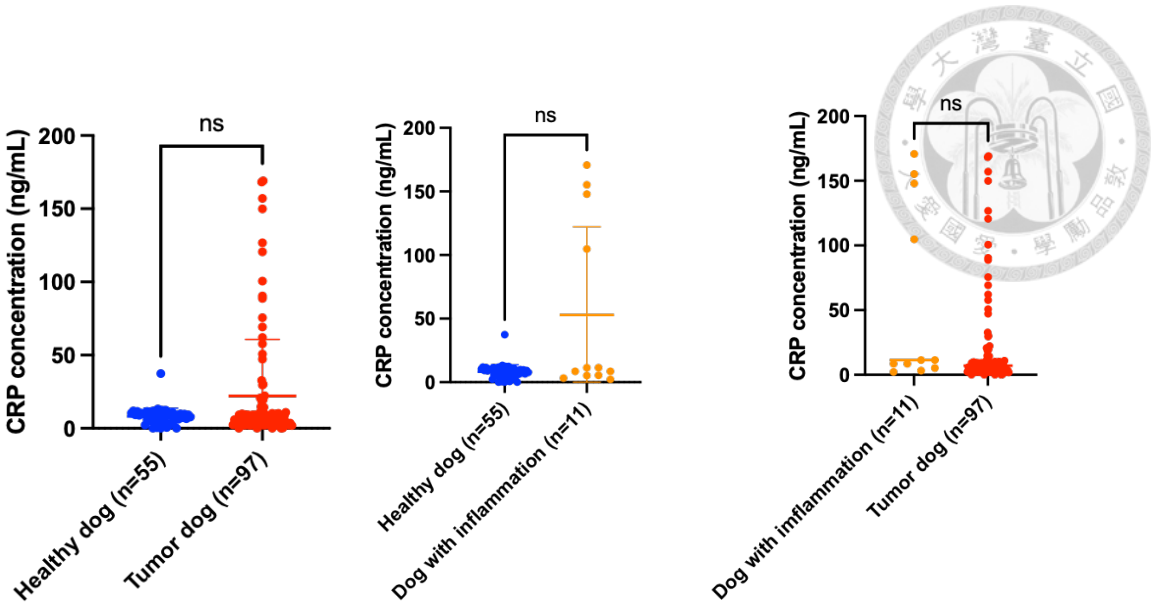

Figure 9. The level of plasma CRP among healthy dogs, dogs with inflammation, and tumor dogs.

There was no statistical significance between the group of healthy dogs and tumor dogs (*P*= 0.7957), healthy dogs and dogs with inflammation (*P*= 0.2342), tumor dogs and dogs with inflammation (*P*= 0.0752).

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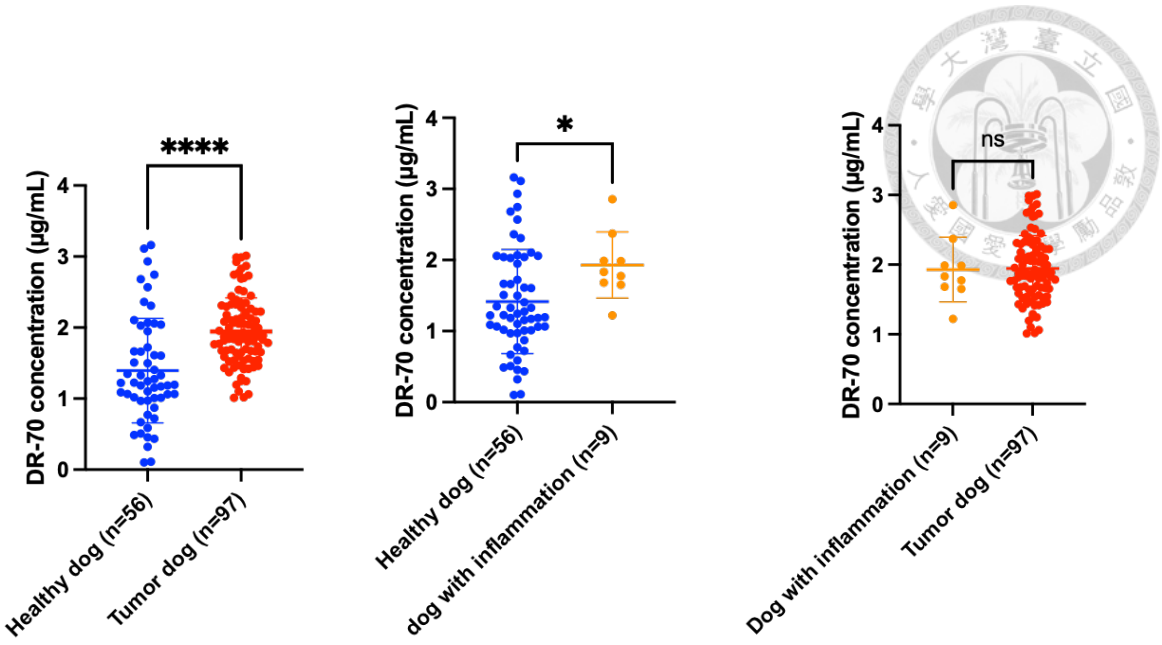

Figure 10. DR-70 levels among healthy dogs, dogs with inflammation, and tumor dogs.

DR-70 levels were significantly higher in tumor ($P< 0.0001$) and inflammatory dogs ($P< 0.0223$), but no statistical significance between dogs will inflammation and tumor dogs ($P= 0.8848$).

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