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Haemoglobin in normal and neoplastic canine mammary glands

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Abstract

Four types of globins for oxygen transport are known in vertebrates, and the haemoglobin is responsible for carrying oxygen in blood. In this study, we found that haemoglobin was also expressed in canine mammary glands. Samples were taken from 26 malignant mammary tumors, 16 normal mammary glands and 10 other normal tissues. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and mass spectrometry were used to investigate haemoglobin in mammary tissues. The results indicated that normal canine mammary glands expressed high levels of haemoglobin protein as shown by Coomassie blue staining. The identity of haemoglobin was confirmed by immunoblotting and mass spectrometry, and the mass spectrometry data revealed that both alpha-haemoglobin and beta-haemoglobin were expressed. Relative to normal mammary glands, the levels of haemoglobin expression in mammary tumors were lower. Our results also indicated that the haemoglobin was endogenously produced in mammary gland tissues and was not derived from the erythroid cells.

Keywords
canine; haemoglobin, mammary gland, mammary tumor, Western blot

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Introduction

Globins are universally important for respiration by reversibly binding oxygen. They are found in diverse organisms including animals, plants, fungi and bacteria.[1,2] Vertebrates have four types of globins for oxygen transport. The tetrameric haemoglobin carries oxygen in blood, and the monomeric myoglobin is responsible for the delivery of oxygen into mitochondria in muscle cells.[3]

The third type of globin, named neuroglobin, is primarily expressed in the brain of man and mouse. Although it is found at relatively low cellular concentration, the high affinity of neuroglobin for oxygen may help oxygen across the blood–brain barrier and supply oxygen to neuronal cells. It has been proposed that neuroglobin in the brain is functionally analogous to myoglobin in muscle.[4,5]

Cytoglobin, the fourth member of globin, has been identified in mouse and man. It is mainly expressed in fibroblasts, liver stellate cells and related cell types. The amino acid sequence of cytoglobin shows about 30% identity with myoglobin, suggesting that cytoglobin and myoglobin have a common ancestor. The function of cytoglobin may be involved in the production of collagen.[5–7]

The amino acid sequences of canine alpha chain and beta chain haemoglobin have been previously elucidated. There are two forms of the alpha chain of canine haemoglobin with a single amino acid difference at residue 130 (threonine or alanine), but only one form of the beta chain.[8,9] The nucleotide sequences of dog neuroglobin and cytoglobin are also available from GenBank (accession numbers AAEX01040503 and AAEX01049981, respectively). Recently, the expression of neuroglobin and cytoglobin in normal canine retinas was investigated; both were widely distributed in

retinal neurons and retinal pigment epithelium, but not in glial cells of the retina.[10]

The aim of this study was to explore the different expression of haemoglobin in the normal and neoplastic mammary glands. To our knowledge, our work is the first report of haemoglobin expressed in the normal mammary gland.

Materials and methods

Animals and tissue samples

Animals involved in this study were 31 dogs (18 mixed-breed dogs, 2 Pomeranians, 2 Akitas, 2 Cocker Spaniels, an Old English Sheepdog, a Shetland Sheepdog, a German Shepherd, a Chihuahua, a Dalmatian, a Beagle and a Wirehaired Fox Terrier). They were referred to the Veterinary Teaching Hospital of National Chung-Hsing University for surgical removal of mammary tumours or routine sterilization. The specimens obtained by surgical biopsy included normal and neoplastic mammary glands, and other normal tissues. Animal tissue was used with permission of the Veterinary Teaching Hospital of National Chung-Hsing University. The specimens were stored in liquid nitrogen for further study. The samples of normal mammary glands were at different stages, including anestrous, proestrous, estrous and metaestrous stages, which were obtained by biopsy from 16 dogs (ranging from 3 months to 12 years old). Among them, five mammary gland tissues were obtained from the same animal with mammary tumours. Other normal tissues obtained from 10 dogs included four normal uterine horns, three lymph nodes, two testicles and one subcutis. Normal blood was also obtained from two healthy dogs. The neoplastic mammary glands were obtained from 26 dogs, which included different tumour stages from stage I (5 dogs), stage II (5 dogs), stage III (4 dogs), stage IV (4 dogs) and stage V (8 dogs); and various tumor types including 3 simple adenomas, 4 benign mixed tumors, 5 simple carcinomas, 11 complex carcinomas and 3 sarcomas. Stages I–V were classified by the modified WHO staging system, that is the TNM system (T = the extent of the primary tumour, N = the status of the regional lymph nodes and M = the absence/presence of distant metastasis).

Pathologic classification of these mammary tumor tissues was determined by histological examination. They were processed by standard histopathological techniques, and hematoxylin-and-eosin-stained sections were examined to assign tumour types according to the WHO-AFIP classification of canine mammary tumors.[11]

Establishment of canine cell line

A 6-year-old female mixed dog presented with a mass size ($11 \times 8 \times 9$ cm) on the abdomen was admitted to the Veterinary Teaching Hospital of National Chung Hsing University in March, 2000. Physical diagnosis suggested that the dog had a mammary gland tumour. The tumour mass was removed surgically. A part of tumour mass ($2 \times 1 \times 2$ cm) was placed on a 10-mm petri dish (NUNC, Roskilde, Denmark) containing 10 mL of DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA), 100 U mL^{-1} of penicillin G and 100 μg mL^{-1} of streptomycin (Hyclone, USA) and then minced into smaller pieces. Three millilitres of Ham's F-12 Nutrient Mixture (Gibco BRL, USA), 100 U mL^{-1} of penicillin G, 100 μg mL^{-1} of streptomycin (Hyclone, USA) and 4 mg mL^{-1} collagenase type III (Gibco BRL, USA) were added into tissue suspensions and then they are incubated at 37 °C for 8 h. After digestions, the suspensions were passed through the filter and centrifuged at 90 g for 10 min. The pellet cells were resuspended and incubated in 9 mL of DMEM and 1 mL of Ham's F-12 Nutrient Mixture supplemented with 10% fetal bovine serum, 100 U mL^{-1} of penicillin G, 100 μg mL^{-1} of streptomycin, 1 μg mL^{-1} of hydrocortisone (Sigma, Steinheim, Germany) and 10 μg mL^{-1} of insulin (Sigma) at 37 °C. To passage the cells, monolayer was washed twice with phosphate buffered saline (PBS) and detached with 0.05%trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution (Gibco BRL, USA) before the eighth passage. After eighth passage, cells were digested with STV buffer (1.37 M NaCl, 0.05 M KCl, 1.06 M glucose, 10 000 U of penicillin G, 10 000 μg of streptomycin, 0.05 M EDTA, 5 g of trypsin and 0.25 mM phenol red) and named DMGT cells.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Each individual specimen (100 mg) was taken from liquid nitrogen and was mixed with 0.5 mL of RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris–HCl pH 7.4, 0.5% sodium deoxycholate, and 0.1% SDS) and ground until an emulsion was formed. Then, 1.5 mL of emulsion was placed on the ice for 20 min, followed by centrifugation at 350 g for 2 min. After removing the lipid-like substance, the pellet was mixed with 0.3 mL of 2× sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue). Protein concentration was determined by using Protein Assay Rapid kit (Wako, Tokyo, Japan) method. Each mixture (~28 μg) was boiled for 10 min before loading into the SDS-12% PAGE and then separated with a Hoefer SE400 electrophoresis apparatus (Hoefer-Amersham-Pharmacia, Giles, UK). The protein was visualized by Coomassie blue staining after electrophoresis. The relative expression scores were measured on SDS-PAGE by computer-assisted program.

Western blotting

Following SDS-PAGE, proteins were electrophoretically transferred onto nitrocellulose membranes. After a blocking step in PBS containing 0.1% Tween-20 (PBST) and 5% dried milk for 1 h at room temperature, the separated proteins on the membrane were probed with rabbit anti-human haemoglobin antibody (Sigma) or rabbit anti-human glycophorin A polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBST-5% dried milk at room temperature for 2–3 h. Subsequently, the filter was washed in PBST four times followed by incubation with horseradish peroxidase-conjugated secondary antibody in PBST-2% dried milk at room temperature for 1 h. After extensive washing, the filters were soaked in Lumino/ Enhancer solution (SuperSignal West Pico chemiluminescent substrate, PIERCE) and exposed to X-ray films.

Mass spectrometry

For the examination of haemoglobin identity, the band at the 15 kDa position on SDS-PAGE was cut off and was subjected to analysis by mass spectrometry, which was conducted by the Mission Biotech (Taipei, Taiwan).

Results and discussion

In this work, high expression levels of haemoglobin in the normal mammary gland were found, and the apparent molecular weights of canine haemoglobin are approximately 15 kDa in SDS-PAGE (Fig. 1). However, the levels of expression of the 15-kDa protein in mammary tumours were lower (Fig. 1).

An immunoblot revealed that the 15-kDa protein was haemoglobin by using an anti-haemoglobin antibody (Fig. 2). Furthermore, the biochemical identity of the canine haemoglobin was verified with mass spectrometry, demonstrating that the 15-kDa band contained both alpha-haemoglobin and beta-haemoglobin (data not shown).

The expression of haemoglobin in various types of neoplastic tissues (benign tumor, simple carcinoma, complex carcinoma and sarcoma) was compared with the normal tissue, and the results showed that the neoplastic tissues were generally lower in haemoglobin expression than the normal mammary gland tissues (Fig. 3A). The degree of expression of haemoglobin in mammary tumors appeared differently in various tumour stages (Fig. 3B).

The differential expression of haemoglobin in normal mammary gland and mammary tumors may have implications for oxygen deficiency in breast cancer. It is estimated that in humans, about 50% of locally advanced breast cancers display hypoxic and/or anoxic tissue areas, which are distributed heterogeneously in the tumour.[12] Our finding (high haemoglobin expression in normal tissue versus low expression in tumour) implies that endogenous haemoglobin can supply more oxygen to the normal mammary gland. The relevance of lower expression of haemoglobin in mammary tumour with hypoxia in tumour remains to be investigated. Thus, the function of haemoglobin in the mammary gland may be similar to that of neuroglobin in neurons for oxygen homeostasis and hypoxia protection.[13]

In addition, we used the DMGT cell (a canine tumor cell line established in our laboratory)

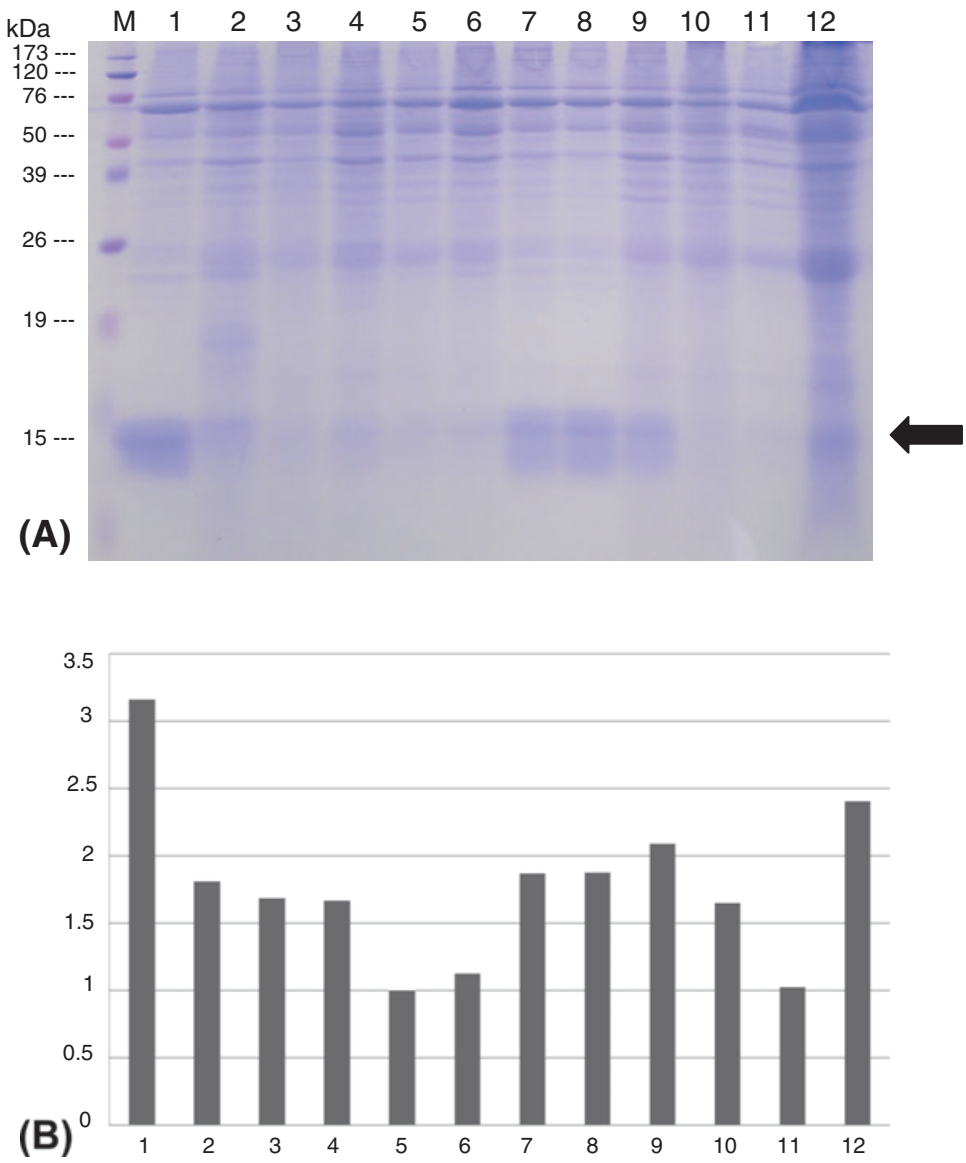

Figure 1. Separation of proteins from mammary tissues by SDS-PAGE. (A) A noticeable band of 15 kDa (indicated by an arrow) was observed in lysate obtained from normal mammary glands (lanes 1, 7, 8 and 9), but not from mammary tumors (lanes 3, 4, 5, 6, 10 and 11). The 15-kDa band containing both alpha-haemoglobin and beta-haemoglobin was verified by mass spectrometry. (B) Quantification of SDS-PAGE in panel A. Lane 1: Normal mammary gland (dog, female, 6 M). Lane 2: Normal mammary gland (dog, female, 3 Y). Lane 3: Mammary tumor (benign mixed tumor), stage II (dog, female, 7 Y). Lane 4: Mammary tumor (complex carcinoma), stage III (dog, female, 9 Y). Lane 5: Mammary tumor (simple carcinoma), stage IV (dog, female, 8 Y). Lane 6: Mammary tumor (complex carcinoma), stage V (dog, female, 12 Y). Lane 7: Normal mammary gland (dog, female, 5 M). Lane 8: Normal mammary gland (dog, female, 3 M). Lane 9: Normal mammary gland (dog, female, 1 Y). Lane 10: Mammary tumor (benign mixed tumor), stage I (dog, female, 7 Y). Lane 11: Mammary tumor (complex carcinoma), stage V (dog, female, 12 Y). Lane 12: Mammary tumor (simple carcinoma), stage V (dog, female, 11 Y).

to examine the level of haemoglobin expression, and it did not exhibit an obvious expression of haemoglobin in the Coomassie blue stained SDS-PAGE (data not shown). This result was basically in agreement with our results of mammary tumors (Figs 1 and 2).

The membrane protein glycophorin A is a characteristic and a major component of erythrocytes.[14]

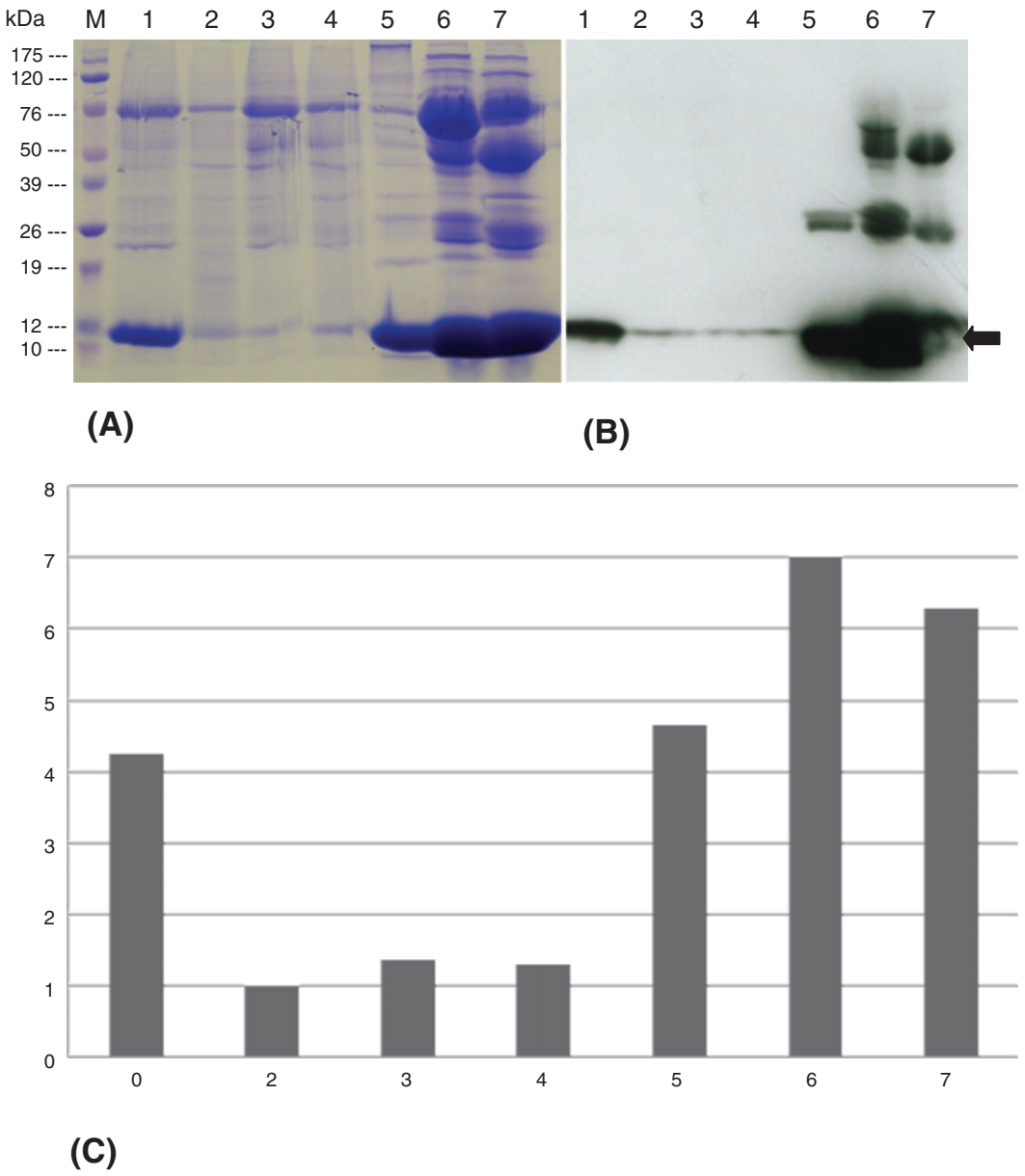

Figure 2. Identification of haemoglobin by Western blotting. Same samples were divided equally for experiments. Results of SDS-PAGE (A) and Western blot (B) using anti-haemoglobin antibody are shown. The position of haemoglobin is indicated with an arrow. Quantification of SDS-PAGE in panel A (C). Lane 1: Normal mammary gland (dog, female, 6 M). Lane 2: Normal mammary gland (dog, female, 3 Y). Lane 3: Mammary tumor (complex carcinoma), stage V (dog, female, 12 Y). Lane 4: Mammary tumor (simple carcinoma), stage IV (dog, female, 8 Y). Lane 5: Blood (pellet) (dog, female, 11 M). Lane 6: Blood (supernatant) (women, 24 Y). Lane 7: Blood (supernatant) (dog, female, 11 M).

To exclude the possibility that most of the haemoglobin seen in our experiments were contributed from the blood of capillaries found in normal mammary gland tissue, we conducted immunoblotting to examine the expression of glycophorin A in the normal mammary gland. As shown in Fig. 4, glycophorin A was not detected in the normal mammary gland by using anti-glycophorin A antibody. Therefore, our results indicated that haemoglobin is endogenously produced in normal mammary gland cells, implying that abundant mammary haemoglobin, in addition

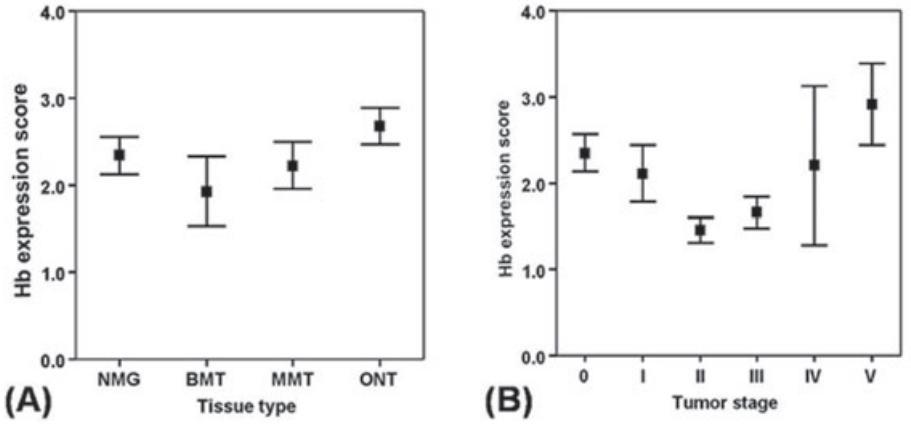

Figure 3. (A) Statistics of haemoglobin (15 kDa) expression scores in normal or neoplastic tissues. Expression level of the 15-kDa protein was quantitatively represented by calculating intensity of bands in SDS-PAGE. NMG: normal mammary glands; BMT: benign mammary tumors; MMT: malignant mammary tumors; and ONT: other normal tissues. (B) Statistics of haemoglobin expression score in various tumor stages. Stage 0 is normal mammary glands. The method for measuring expression scores is same as that of (A). Each full square indicates an average score, and each bar represents the mean of standard error.

to oxygen transport, may have unidentified physiological functions.

The same surgical procedures were performed for normal mammary gland and mammary tumor; hence, the basal levels of haemoglobin accidentally from capillaries would be similar. The same surgical procedures of dogs minimize the fluctuation of haemoglobin contamination.

It was shown that haemoglobin was expressed in alveolar epithelial cells of rats[15] and in human and rodent cells.[16] These two studies detected haemoglobin by using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunological methods, whereas the haemoglobin in canine mammary gland could be observed by Coomassie blue staining in our results. Consequently, it can

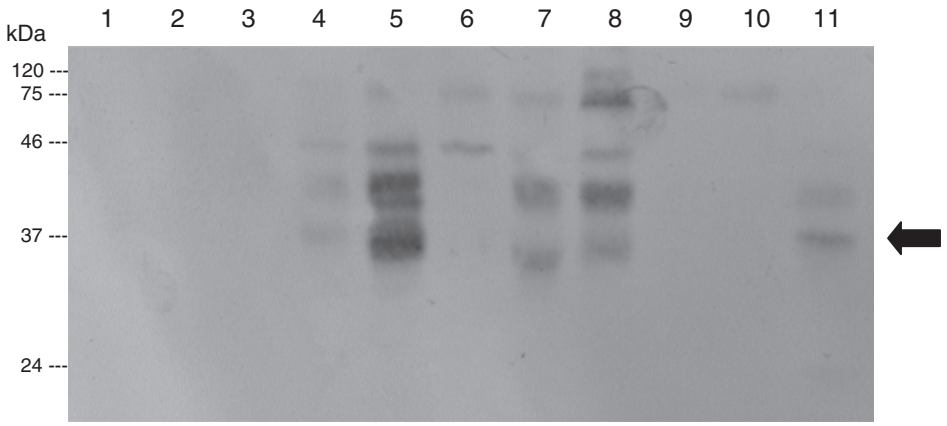

Figure 4. Western blot of glycophorin A. The expression of glycophorin A was not detectable in the normal mammary gland and tissues (lanes 1, 2, 9 and 10). Lane 11 was the expression of glycophorin A in erythrocytes, serving as a positive control. For tumors, the levels of glycophorin A expression were variable; and bands of high molecular weights because of cross reaction were observed (lanes 5, 6 and 7). Lane 1: Normal mammary gland (dog, female, 6 M). Lane 2: Normal mammary gland (dog, female, 3 Y). Lane 3: Mammary tumor (benign mixed tumor), stage II (dog, female, 7 Y). Lane 4: Mammary tumor (benign mixed tumor), stage I (dog, female, 7 Y). Lane 5: Mammary tumor (simple carcinoma), stage IV (dog, female, 12 Y). Lane 6: Mammary tumor (complex carcinoma), stage V (dog, female, 12 Y). Lane 7: Mammary tumor (simple carcinoma), stage V (dog, female, 11 Y). Lane 8: Testicle (dog, male, 1 Y). Lane 9: Normal uterine horn (dog, female, 6 M). Lane 10: Normal skin (dog, Male, 6 Y). Lane 11: Blood [dog (Elmo), female, 11 M].

be concluded that the amount of haemoglobin in mammary gland was more plentiful than human and rodent lung cells. All these findings indirectly implicated that haemoglobin plays not only oxygen-transport role in erythroid cells but also non-oxygen-carrying role in other types of cells.

The functions of microbial and invertebrate haemoglobins can defend against oxidative and nitrosative attack, whereas haemoglobins of mammals mediate the metabolic coupling of oxygen transport with tissue demand. In recent years, it is shown that haemoglobin and other proteins of oxygen transport are also capable of binding nitric oxide (NO), which plays roles in host defense.[17,18] In addition, it is generally thought that the predominant roles of a globin ancestor were binding with oxygen and oxygen-derived species (for examples, NO and CO), serving for elimination of these molecules by enzymatic activity. Later, globin may have functioned in oxygen and nitric oxide transport and could have been an important specialized development leading to the emergence of metazoans following the rise of earth oxygen levels.[2] Therefore, new *in vivo* roles of haemoglobin may continue to emerge in the future.

Furthermore, it was found that nitric oxide, competing with oxygen, can influence mitochondria by reversible interaction with the cytochrome c oxidase, and the consequence of inhibiting mitochondrial respiration by nitric oxide can cause metabolic hypoxia.[19] It would also be of interest to study the relationship between haemoglobin, nitric oxide and canine mammary tumors.

The potential non-oxygen-carrying function of haemoglobin as well as the mechanism of haemoglobin gene stimulation in cells of the mammary gland need further studies, but our present finding may be of significance for the physiological and pathophysiological implications of mammary tissue. In summary, haemoglobin was highly expressed in the normal mammary gland. Results also showed that haemoglobin was endogenously produced in the normal mammary gland and was not derived from erythroid cells.

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