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Haemoglobin Epsilon as a Biomarker for the Molecular Detection of Canine Lymphoma

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A B S T R A C T

Lymphoma is a cancer arising from B or T lymphocytes that are central immune system components. It is one of the three most common cancers encountered in the canine; lymphoma affects middle-aged to older dogs and usually stems from lymphatic tissues, such as lymph nodes, lymphoid tissue, or spleen. Despite the advance in the management of canine lymphoma, a better understanding of the subtype and tumor aggressiveness is still crucial for improved clinical diagnosis to differentiate malignancy from hyperplastic conditions and to improve decision-making around treating and what treatment type to use. This study aimed to evaluate a potential novel biomarker related to iron metabolism, embryonic haemoglobin (*HBE*), for early diagnosis. Archived samples in combination with prospective samples collected from dogs with and without lymphoma were used in this study for the retrospective analyses of this tumor based on the same biomarker amplified by real-time quantitative polymerase chain reaction. The *HBE* mRNA was aberrantly expressed in canine B and T cell lymphoma compared to the normal lymph node tissue and hyperplastic lymph nodes. In conclusion, this study identified a novel potential biomarker for improving lymphoma diagnosis and treatment in dogs. Further studies with larger sample sizes are needed to confirm the suitability of this biomarker for canine lymphoma diagnosis.

Keywords: canine lymphoma, embryonic haemoglobin, subunit epsilon, RT-qPCR

INTRODUCTION

The small protein called globin, known as haem, plays a crucial role in various biological functions and can be found in all life kingdoms (1,2). It can be categorized into four main groups: cytoglobin, neuroglobin, myoglobin, and haemoglobin (Hb), each with distinct functions and distribution in tissues (3). During development, starting from the yolk sac blood islands, Hb in erythroid cells undergoes a series of transitions, changing from embryonic to fetal (primitive) and finally to adult (definitive) Hb in humans. In mice, the transition occurs directly from embryonic to adult Hb (4). In humans, the embryonic Hb is replaced by fetal haemoglobin by the end of the first trimester. At birth, approximately 50% of human haemoglobin is fetal, and by 12 months of age, the haemoglobin composition resembles that of adults, with only a very small amount of fetal haemoglobin remaining.

Haemoglobin is composed of two α-like globins (embryonic ζ or adult α) and two β-like globins (embryonic

ε, fetal γ, or adult δ/β in humans; embryonic βh1/εY or adult βmaj/βmin in mice), forming a tetramer (5, 6). In humans, these tetramers consist of embryonic haemoglobin s Hb Gower 1 (ζ2Ɛ2), Hb Gower 2 (α2Ɛ2), and Hb Portland 1 (ζ2γ2), fetal haemoglobin HbF (α2γ2), and adult haemoglobins HbA (α2β2) and HbA2 (α2δ2), accounting for approximately 97% and less than 3% of adult Hb, respectively (7).

Physiologically, embryonic and foetal Hbs (HbF/HBE, 2 alpha and 2 gamma chains, α2γ2), as well as adult HbA2 have higher oxygen affinity than the HbA. Mammalian Hb functions include the transportation of gases and scavenging oxidizing agents (8, 9) to protect cells from oxidative and nitrosative stress (10, 9). Notably, Hb or subunits thereof have been revealed to be ectopically expressed in newly formed tumor blood vessels and the interstitium between tumor cells in neuroblastoma and retinoblastoma (11). In children with leukaemia, increased circulating levels of HbF have also been associated with a worse outcome (12).

As in humans, HbA is the most common Hb type in canine species (13). Haemoglobin has been studied from the perspective of canine disease, comparative genomics, and protein structure (14, 15). Although Hb has 100% sequence identity among dog breeds, greyhound Hb has been found to have higher oxygen affinity than that of other dogs (16, 17). To date, only one published study has investigated Hb expression in canine cancer. This study found upregulation of Hb expression in normal versus neoplastic dog mammary glands and identified that the neoplastic mammary gland endogenously produces Hb rather than production derived from erythrocytes (14). To date, no published reports have investigated Hb expression in canine lymphoma.

Previous unpublished work from Murdoch University has confirmed the expression of HBE in canine lymphoma using microarray data. Hence, this study aimed to investigate these findings further to explore the role of the embryonic haemoglobin gene (*HBE*) in canines by studying its expression in canine nodal lymphoma by real-time quantitative polymerase chain reaction (RT-qPCR).

MATERIALS AND METHODS

Sample Collection

We collected a total of 153 samples from dogs for this study, including both frozen samples of total RNA and tissue sections preserved in formalin-fixed paraffin- embedded (FFPE) format. These samples were obtained from canine cases enrolled in the study either retrospectively or prospectively between the years 2006 and 2019. Among these cases, there were 129 cases of lymphoma, 8 cases of hyperplastic lymph nodes, and 16 cases of normal lymph nodes.

For the prospective enrollment, we recruited canine patients from two different veterinary practices: one located in Australia called Perth veterinary specialists and the other in Italy known as Clinical Veterinary Malpensa. During the diagnostic process at the time of presentation, we also assessed fine needle aspirate material from the same group of patients to evaluate *HBE* expression using RT-qPCR. We had access to a substantial amount of archived material stored in the histopathology block bank at Mudoch University, including frozen lymph node biopsies from 102 dogs diagnosed with lymphoma.

Total RNA Extraction, Quantification, Qualification and cDNA Synthesis

Total RNA was extracted from both the FFPE lymph node samples and RNALater (surgical biopsies and FNA) samples where possible. For FFPE samples (5 × 10 mm thick sections), total RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, USA) according to the manufacturer's instructions with minor modifications. The modifications made included extra xylene washes (3×) for blocks with a high paraffin to tissue ratio, proteinase digestion for 4.5 h and reduced elution volume to 40 µL for concentrating the RNA due to the lower mass of tissue. For samples stored in RNALater (QIAGEN, USA) solution, total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, USA) to the aqueous phase, and purification of the aqueous phase by the PureLink™ Mini kit was done. Maxima™H Minus cDNA Synthesis Master Mix with dsDNase was used for cDNA synthesis from all RNA samples. Random decamers (2 µL of 10 µM per reaction) supplied as 50 µM were added to the priming reactions with FFPE samples to improve cDNA synthesis as the FFPE RNA samples can be damaged by fragmentation (18). Nanodrop spectrophotometry (NanoDrop ND-1000, USA), denaturing agarose gel electrophoresis, and Agilent 2100 Bioanalyzer spectrophotometry (Agilent Technologies, Germany) were used to evaluate RNA quantity and quality before performing cDNA synthesis and RT-qPCR.

Primer Selection and Optimization of RT-qPCR Protocols

Primers specific for *HBE* were designed using Perlprimer. A search was conducted in Primer-BLAST to include all known canine HBE variants to confirm primer specificity (19). Two endogenous reference genes were also chosen for RT-qPCR normalization; these genes were ribosomal protein L19 (*RPL19*) (20-22) and phosphoglycerate kinase 1 (*PGK1*) (23). All primers used in this study for *HBE*, *RPL19*, and *PGK1* were obtained from ThermoFisher Scientific and are detailed in Table 1. The RT-qPCR reactions were performed with the study samples in triplicate and comprised of PowerUp™ SYBR™ Green

Master Mix, forward and reverse primers (300 nM each), and cDNA template. The amount of cDNA template used was 5 ng for the RNALater samples and 10 ng for the FFPE samples. The reference or control sample for qPCR reactions was a pool of cDNA from normal lymph nodes.

The aforementioned genes were partially amplified in a total volume of 25 µL using a QuantStudio 6 FlexReal-time PCR System, software v1.3 in Fast-96 well (0.1 mL) plates. Thermocycling conditions were as follows: Uracil DNA Glycosylase (UDG) activation at 50 °C (2 min), UDG and DNA polymerase denaturation at 95 °C (10 min), amplification for 40 cycles at 95 °C (15 sec) and 57 °C (1 min). Then, extension at 72 °C (1 min) followed by dissociation curve analysis at 62 °C (45 sec) with gradient 0.05 °C/sec to 95 °C. Gene expression analysis amongst groups across multiple assays was done using Expression Suite Software (version 1.0.4).

Table 1. Forward and reverse primers' sequences and amplicon length for *HBE1*, *RPL19*, and *PGK1*.

Gene	Primer Sequence	Amplicon length
HBE1	Forward: 5'-CACGTGGATCCCGAGAACTT-3' Reverse: 5'-CAACACCAGCCACCAGTTTC-3'	124 bp
RPL19	Forward: 5'-CTCGAATGCCTGAGAAGGT-3' Reverse: 5'-CTATGATACATGTGGCGGTC-3'	105 bp
PGK1	Forward: 5'-CATCATAGGTGGTGGAGAC-3' Reverse: 5'-AGGACTTTACCTTCCAAGAG-3'	116 bp

Unique and pooled cDNA from RNA extracted from B cell lymphoma (sample L19) and normal lymph node tissue were used for primer optimization using the PowerUp™ SYBR™ Green Master Mix kit according to the 'manufacturer's instructions. The primer concentration range used was 300-800 nM with 1-10 ng cDNA template. A standard curve was produced by serial dilutions of the pooled control and L19 cDNA (1:10, 1:20, 1:40, and 1:80) to determine the combination of primer concentration and dilution of the cDNA template best suited to the study. The optimal annealing temperature and sample concentration were determined for maximum efficiency (86-100%) of RT-qPCR assays for the two reference genes (*RPL19* and *PGK1*) and the target gene (*HBE1*). The standard curve analysis involving linear regression, amplification curve, and melt peak analysis of the PCR products was used to select the annealing temperature.

Statistical Analysis

Analyses were conducted using Expression Suite Software (version 1.0.4 and SPSS v.24, IBM Corp.). A *P*-value equal to or less than 0.05 was considered significant in all cases. Normality was assessed with the Shapiro-Wilk test and a visual inspection of histograms and Q-Q plots. The differential *HBE1* gene expression was compared among groups using the Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests to identify group differences.

Results

Primer Optimisation

Figure 1 displays the analysis of the standard curve linear regression at 57 °C annealing temperature. The HBE primers had an amplification efficiency of 100.656% with R^2=0.903 (Figure 1). The amplification efficiencies of the reference genes primers (*RPL19* and *PGK1*) were 97.88% (R^2=0.996) and 85.613% (R^2=0.996), respectively (Figure 2).

The amplification plots for these genes are illustrated in Figures 3 and 4, revealing the association between the sample concentration represented by ΔRn (the magnitude of normalized fluorescence signal) and cycle threshold (CT) within the number of cycles. The *HBE1* gene showed an amplification plot with a CT between 27-34 cycles; this demonstrates the early exponential phase to the plateaued phase of maximum fluorescence according to the dilution factor of the serial standard curve (Figure 3). The *RPL19* gene showed the ideal amplification plot with a CT between 16-20 cycles. This indicates the early exponential phase to the plateaued phase of maximum fluorescence according to the dilution factor of the serial standard curve (Figure 4a). The *PGK1* gene showed the amplification plots of 20-24 CT values of the early exponential phase to the definite plateau of fluorescence at the maximum point (Figure 4b).

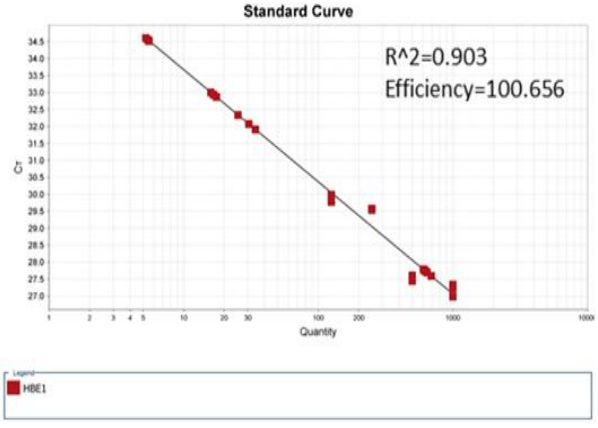

Figure 1. Standard curve of the target gene (*HBE1*) amplified by RT-qPCR using 4 concentrations (1:10, 1:20, 1:40, and 1:80) of L19 cDNA combined with L6, L42, L44, and L67 as unknown cDNA samples. The RT-qPCR reactions were performed in triplicate for each cDNA sample. The linear regression of *HBE1* represented a reaction efficiency of 100.656% and a correlation coefficient of 0.903. The CT values of the replicates were within 0.5 in the standard curve.

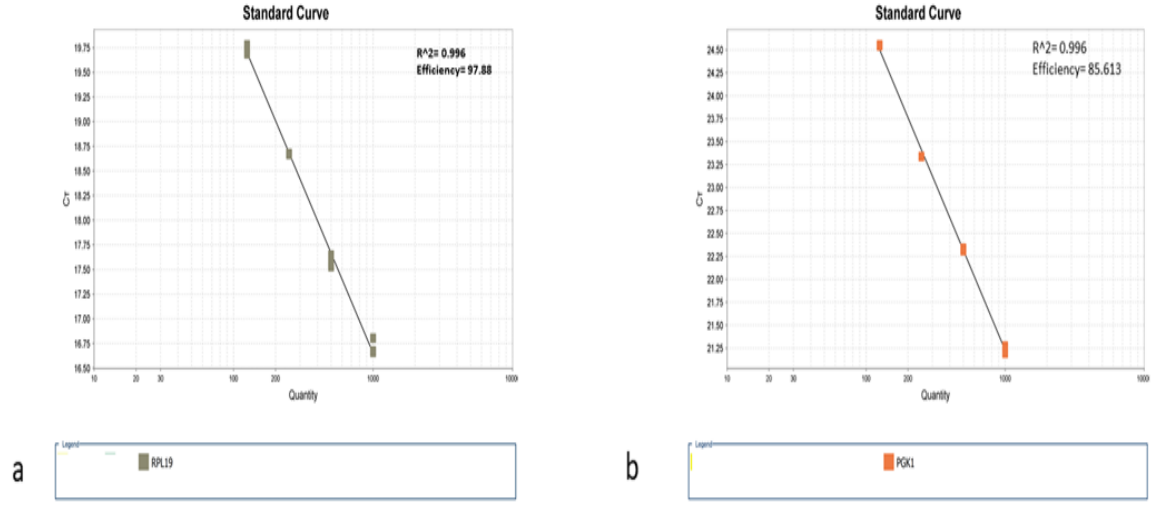

Figure 2. Standard curves of the reference genes (*RPL19* and *PGK1*) amplified by RT-qPCR using 4 concentrations (1:10, 1:20, 1:40, and 1:80) from the pooled normal control lymph node cDNA. The RT-qPCR reactions were performed in triplicate. (a) *RPL19* had a reaction efficiency of 97.880% and a correlation coefficient of 0.996. (b) *PGK1* had a reaction efficiency of 85.613% and a correlation coefficient of 0.996.

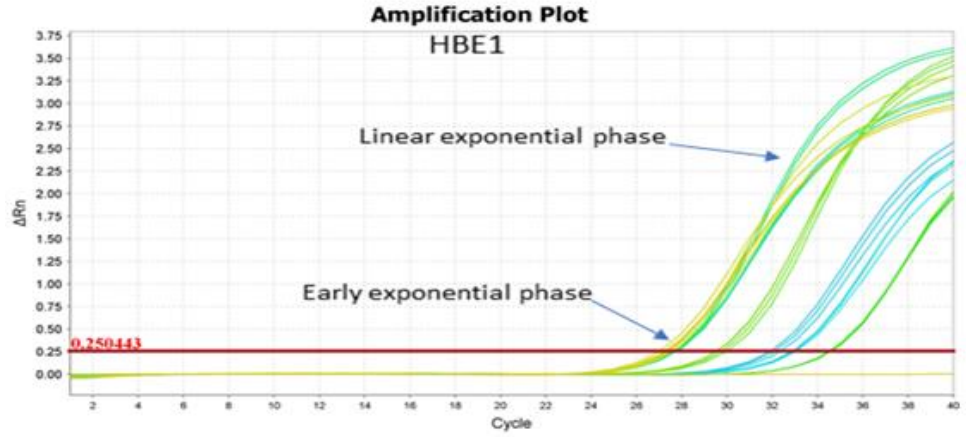

Figure 3. Amplification plots of the RT-qPCR were used to amplify the target gene (*HBE1*) in different dilution factors (1:10, 1:20, 1:40, and 1:80) of L19 and pooled normal lymph nodes cDNA. The CT values of the amplified *HBE1* were between 27-34 cycles. The early exponential phase and the plateau phase of maximum fluorescence are shown on the amplification plot. All triplicate samples were separated as a single band based on the dilution factor for the plot

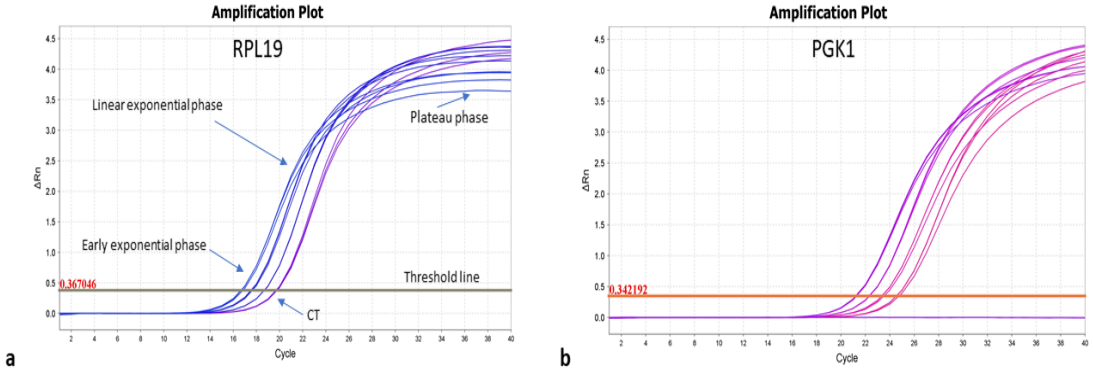

Figure 4. Amplification plots of the RT-qPCR were used to amplify the reference genes (*RPL19* and *PGK1*) in different dilution factors (1:10, 1:20, 1:40, and 1:80) of pooled normal lymph node cDNA. **(a)** The CT values of the amplified *RPL19* were between 14-20 cycles. The early exponential phase and the plateau phase of maximum fluorescence are shown on the amplification plot. **(b)** The CT values of the amplified *PGK1* were between 20-24 cycles

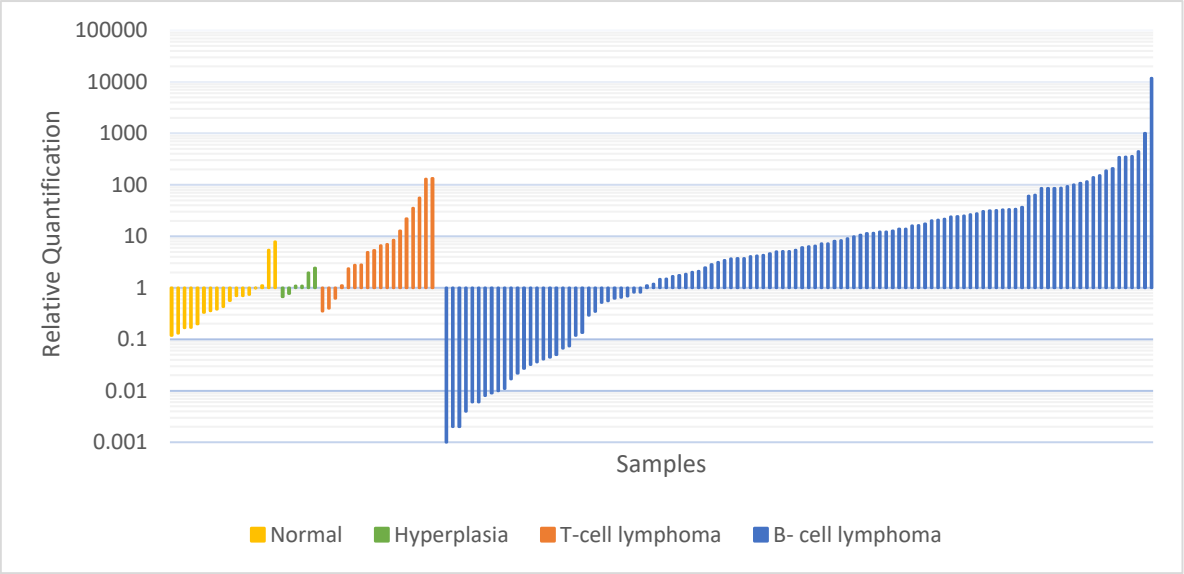

Figure 5. Histogram displaying the *HBE1* gene expression in normal lymph nodes (yellow columns), reactive lymph nodes (green columns), T cell lymphoma (orange columns), and B cell lymphoma (blue columns).

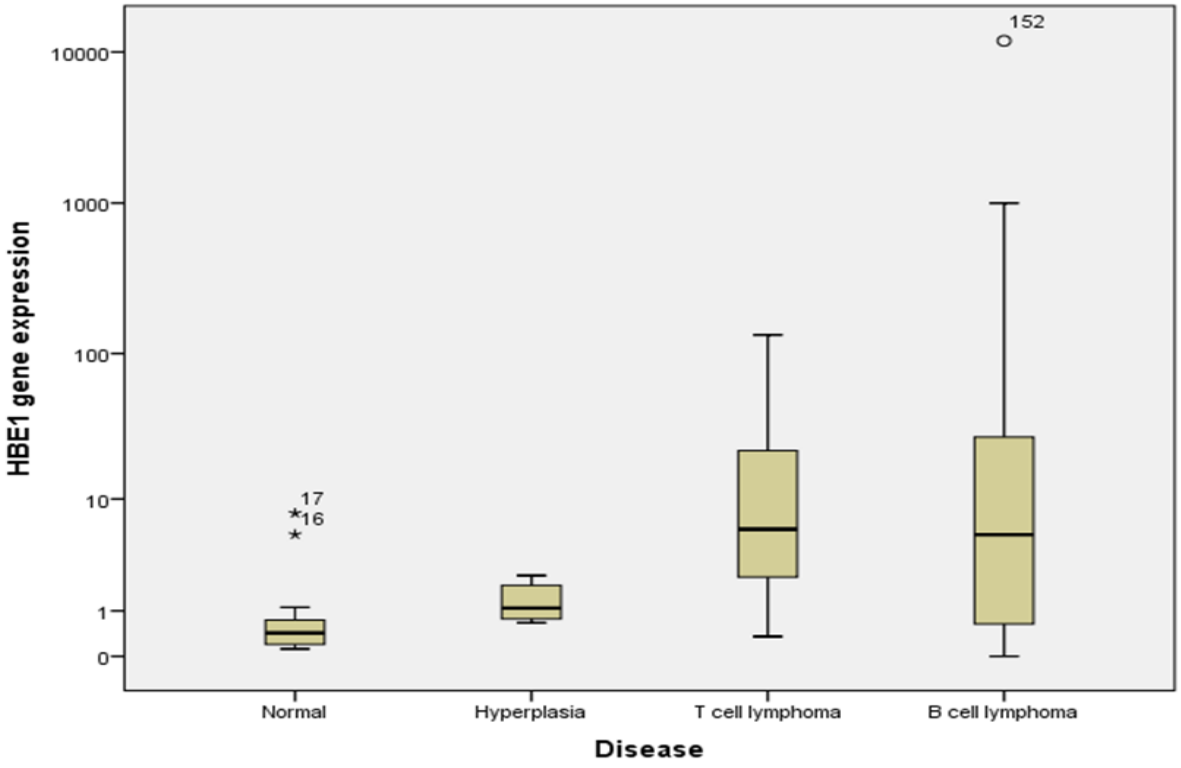

Figure 6. Box and whisker plots show statistically significant differences in the *HBE1* mRNA gene expression among normal lymph nodes, reactive lymph nodes, T-cell lymphoma, and B-cell lymphoma. The *HBE1* gene expression was higher in lymph nodes from dogs with T-cell lymphoma compared to normal lymph nodes (P=0.001). Similarly, expression of the same gene was also higher in lymph nodes from dogs with B-cell lymphoma compared to normal lymph nodes (P=0.002). However, its expression was not significantly different between B and T cell lymphoma or between B or T cell lymphoma and hyperplastic lymph nodes. For each group, the boundaries of the box indicate the first and third quartiles, such that the length of the box is the interquartile range (IQR). The horizontal line within the box indicates the group median. The ends of the whiskers represent 1.5*IQR. Open circles represent outliers above 1.5*IQR, while stars represent extreme outliers (>3*IQR). The numbers associated with the outliers are the sample identification numbersfrom

The *HBE1* Gene Expression

A range of the *HBE1* gene expression was identified by RT-qPCR for the samples of the normal lymph nodes, hyperplastic lymph nodes, and those with T cell and B cell lymphoma (Figure 5). The relative *HBE1* gene expression was significantly different when compared among the four groups (Figure 6). The median *HBE1* gene expression value in the normal lymph node group was 0.43 (range = 0.12-7.9) compared to 1.10 (range = 0.67-2.43) for reactive lymph nodes, 5.96 (range = 0.35-133.06) for T cell lymphoma, and 5.41 (range = 0.00-11861.76) for B cell lymphoma. Pair-wise comparisons revealed significantly higher *HBE1* gene expression in B cell lymphoma compared to the normal lymph nodes (P=0.002) and significantly higher *HBE1* gene expression in T cell lymphoma compared to the normal lymph nodes (P=0.001). There was no significant difference in the *HBE1* gene expression between lymph nodes from dog with B versus T cell lymphoma or between hyperplastic lymph nodes and B or T cell lymphomas.

DISCUSSION

The expression of haemoglobin epsilon gene in canine lymphoma has not been previously documented. However, in this study, a technique called RT- qPCR was used to examine the mRNA of an iron -related marker called *HBE1*. It was discovered that this marker was expressed abnormally in canine B- and T-cell lymphoma compared to normal lymph node tissue and hyperplastic tissue. This finding is quite interesting because it suggests that acquired function of binding oxygen, through the abnormal expression of *HBE1*, may be present in canine lymphoma. It is worth noting that the ectopic expression of haemoglobin has been associated with some types of cancer in the past, as indicated by a study conducted by Chudwin et al. in 1977. They observed a significant increase in fetal haemoglobin in a subset of human cancer patients. Particularly those with leukemia, multiple myeloma, testicular tumors (24), and lymphoma, which is relevant to our investigation.

This association is not entirely surprising since tumors often experience hypoxia and oxidative stress during their development, as reported in epithelial tumors (25), glioblastoma (26), and diffuse large B-cell lymphoma (DLBCL) (27).

Further investigations into the abnormal expression of globins in solid tumors and cancer cells have demonstrated an increase in expression, particularly in epithelial cancers and breast cancer, where the cancerous cells originate from epithelial tissues (25, 28). Notably, haemoglobin has also been detected in cervical carcinoma cells, suggesting its role as an antioxidant that mitigates oxidative stress-induced damage in this specific type of cancer cells (29). As a result, the up-regulation of the *HBE1* gene in canine T- and B-lymphoma indicates the potential for enhanced oxygen availability and/or the capacity to cope with oxidative stress within the tumors. It is worth mentioning that HBE exhibits a higher affinity for oxygen compared to adult Hb.

Overall, our finding suggests that ectopic expression of globins may have relevance to canine lymphoma biology in terms of allowing tumor cells to acquire the ability to exist in situations of restricted oxygen supply and/or deal with oxidative stress, and further studies to investigate this are warranted.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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[١]مدرسة الطب البيطري، جامعة مردوك، ببرث، غرب استراليا ص.ب ٦١٥٠، استراليا، [٢]اختصاص العلوم الطبية، الجزيئية والجنائية، جامعة مردوك، ببرث، غرب استراليا ص.ب ٦١٥٠، استراليا، [٣]فرع الفسلجة والكيمياء الحياتية والادوية، كلية الطب البيطري، جامعة بغداد، بغداد، العراق، [٤]قسم مركز بيرث البيطري للمتخصصين، بيرث، غرب استراليا ص.ب ٦٠١٧، استراليا

الخلاصة

سرطانة الغدد اللمفاوية هو ورم خبيث ينشا من الغدد اللمفاوية (خلايا T, B) التي تعتبر من مكونات مركز الجهاز المناعي ويعتبر واحد من ثلاثة سرطانات شائعة في الكلاب والذي يصيب الاعمار المتوسطة والكبيرة وعادة ما ينشا من الانسجة اللمفاوية مثل الغدد اللمفاوية والنسيج اللمفاوي او الطحال. على الرغم من التطور الكبير في كيفية ادارة هذا المرض و التعامل معه الا ان فهم تطوره الى مراحل عدوانية بات مهما وذلك لتحسين التشخيص السريري من اجل تفريق هذا الورم الخبيث عن فرط التنسج وذلك لاتخاذ القرار المناسب للعلاج. هدفت هذه الدراسة الى تقييم المعلم الحيوي, الهيموكلوبين الجنيني, الجديد وغير المالوف والذي له علاقة في ايض الحديد في تشخيص هذا النوع من الاورام الخبيثة. ولتحقيق ذلك ,تم استخدام عينات مخزونة بالاضافة الى عينات جمعت انيا من كلاب مصابة وغير مصابة بسرطان الغدد اللمفاوية في تشخيص الهيموكلوبين الجنيني باستخدام تقنية تفاعل البولميرز المتسلسل (النسخ العكسي). وقد وجد الهيموكلوبين الجنيني بصورة غير طبيعية في سرطانة الغدد اللمفاوية مقارنة بالعينات غير المصابة. نستنتج من ذلك ان الهيموكلوبين الجنيني ممكن استخدامه في تحسين تشخيص وعلاج سرطانة الغدد اللمفاوية في الكلاب ولاثبات تلك النتيجة يحتاج الى دراسات جديدة وبحجم عينات سريرية اكبر.

الكلمات المفتاحية: سرطانة الغدد اللمفاوية للكلاب، الهيموكلوبين الجنيني، ايبسلون، تقنية تفاعل البولميرز المتسلسل (النسخ العكسي)