Received: 29 August 2016 | Revised and accepted: 20 December 2016

DOI: 10.1111/vco.12304

WILEY Veterinary and Comparative Oncology

ORIGINAL ARTICLE

Characteristics of extracellular cyclic AMP-dependent protein kinase as a biomarker of cancer in dogs

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Funding information
Biattic, Grant/Award number: S-2016-0517-000; NRF-2015-Global PhD Fellowship Programme of the National Research Foundation of Korea (NRF); Korean Government, Grant/Award number: NRF-2015H1A2A1034135.

Objective: Early and proper diagnosis of cancer is the most critical factor for the survival and treatment of veterinary cancer patients. In this study, we evaluated extracellular cyclic AMP-dependent protein kinase A (ECPKA) level in serum as a useful cancer biomarker in dogs.
Methods: ECPKA levels were detected in sera from dogs with cancers (n = 48), benign tumours (n = 18), and non-tumour diseases (n = 102) as well as healthy control dogs (n = 54) utilizing enzyme-linked immunosorbent assay (ELISA).
Results: Sera from dogs bearing various types of cancer exhibited markedly increased levels of ECPKA by up to 7.1-, 8.8-, and 10.9-fold compared with those from dogs harbouring benign tumours, dogs with non-tumour diseases, and healthy control dogs, respectively (P < .0001). In addition, serum ECPKA level did not show statistically significant correlation with gender, breed, or age of dogs or their non-cancerous disease conditions.
Conclusion: Our data strongly propose that detection of serum ECPKA level is a potential and specific diagnostic tool for cancer in dogs.

KEYWORDS

cancer biomarker, dog, enzyme-linked immunosorbent assay, extracellular protein kinase A, serum

1 | INTRODUCTION

Early and proper diagnosis of cancer is the most critical factor for patient survival and the treatment of cancer. Therefore, for decades, many efforts have been focused on the identification of diagnostic biomarkers for early detection of malignant neoplasia in veterinary medicine.[1]

Cyclic AMP (cAMP)-dependent protein kinase A (PKA), a serine/threonine protein kinase mediating cAMP action in mammalian cells, is involved in controlling various biological processes such as cell proliferation, differentiation, metabolism and apoptosis.[2,3] Deregulation of PKA has been linked with the initiation and progression of cancer, and its overexpression is frequently observed in different types of human cancer.[2,3] Hence, PKA has been suggested as a potential molecular target for a diagnostic biomarker in human cancer.

Inactive PKA holoenzyme is a tetramer composed of 2 catalytic and 2 regulatory subunits.[3] Upon binding with cAMP on regulatory

subunits, the inactive PKA tetramer is dissociated into 1 dimer of regulatory subunits and monomers of active catalytic subunits, which then phosphorylate various target proteins in both nucleus and cytoplasm.[4] Four isoforms of regulatory subunits, RIα, RIβ, RIIα and RIIβ, have been identified through biochemical studies, and outcomes of PKA signalling activation have been shown to depend on the types of regulatory subunit isoforms in cells.[4] The expression of RII isoforms is preferentially observed in normal tissues and inhibits the growth of cells, whereas the expression of RI isoforms (RIα/PKA-I) stimulates cell proliferation. In particular, overexpression of the RIα/PKA-I is highly correlated with cancer progression, multidrug resistance and various types of cancer patients with a poor prognosis.[5–12]

Interestingly, PKA is expressed mostly intracellularly in normal cells, whereas an extracellular form of the PKA (ECPKA) is secreted from numerous types of human cancer cell lines including prostate, bladder, breast, colon carcinoma and lung adenocarcinoma.[3,10,12] In addition, ECPKA is detected at high concentrations in sera from human patients with various types of cancer but not in sera from healthy volunteers.[12] Furthermore, it has also been shown that the serum level of ECPKA decreases after surgical resection of the tumour in human melanoma patients.[11] These observations raise the possibility that ECPKA in serum could be a valuable diagnostic and prognostic biomarker for human cancer.[11] Considering that ECPKA is detected in other mammalian cancer cell lines such as mouse adrenocortical carcinoma cells,[10] ECPKA is expected to be detected in sera from cancer-bearing mammals other than humans. However, it remains to be determined whether ECPKA is a useful biomarker for detecting cancers in other animals as well as in humans.

In veterinary practice, several effective biomarkers, including nucleic acids, metabolites, proteins or enzymes produced and released by cancer cells as well as cytokines, have been investigated and suggested for diagnosis and prognosis of cancer in dogs.[1,13] Among those cancer biomarkers, thymidine kinase 1 (TK1), C-reactive protein (CRP), α1-acid glycoprotein (AGP) and haptoglobin have been most extensively evaluated for their correlation with cancer. Measurement of serum TK1 level has been proven to be a useful diagnostic tool for detection of various types of malignant tumours in dogs as well as prediction of clinical outcomes of anti-cancer therapy in lymphoma patients.[13–18] However, additional measurement of CRP, a widely accepted biomarker of inflammation, in serum is necessary to improve the limitation of TK1 activity assessment for detecting solid tumours other than hemangiosarcoma in dogs.[19] In addition, although CRP, AGP and haptoglobin have been shown to be markedly increased in sera from dogs with neoplasia and thus potential biomarkers for cancer detection in dogs,[13,17–24] they are also detected at high levels in sera from dogs with severe inflammatory and infectious diseases.[25–27] Hence, measurement of these acute phase proteins for detecting cancer can be affected by various non-cancerous conditions. Indeed, it was reported that increased serum level of CRP in dogs with mammary carcinoma is associated with skin ulceration but not with the carcinoma itself.[28] Furthermore, to date, few biomarkers have been proven useful for distinguishing malignant tumours from benign tissues and tumours in dogs. Thus, the identification of a novel biomarker, which can discriminate cancer from inflammation or non-cancerous diseases as well as benign tumours, and the evaluation of its clinical usefulness will have significant implications for the diagnosis and prognosis of cancer in canine patients.

Although accumulating evidence indicates that ECPKA is a potential diagnostic target for cancer detection in mammals, the correlation of ECPKA serum level in dogs with cancer has never been investigated. Here, we examined whether ECPKA could be detected in sera from dogs with cancers including carcinomas, sarcomas and hematopoietic cancers and validated its utility as a potential biomarker for cancer detection in dogs. We speculate that ECPKA secretion from cancer cells might be useful for cancer detection in dogs.

2 | MATERIALS AND METHODS

2.1 | Serum samples

Serum samples were collected from dogs diagnosed with cancer (n = 48), benign tumours (n = 18) or non-tumour diseases (n = 102) as well as healthy controls (n = 54) using serum separator tubes within 1 hour of blood collection at licenced universities and veterinary clinics and stored at −80°C until used. All dogs with cancer and benign tumours were diagnosed by histopathology (n = 20 and 10, respectively), cytology (n = 21 and 4, respectively) or both (n = 7 and 4, respectively). The diagnosis of non-tumour diseases was made on the basis of complete blood count (CBC), serum chemistry, X-ray examination and ultrasound imaging followed by specific diagnostic tests such as adrenocorticotropin (ACTH) stimulation test, Coombs test, bacterial and fungal culture, echocardiogram, and magnetic resonance imaging (MRI). Healthy dogs were determined to be healthy based on no remarkable finding in CBC, serum chemistry and X-ray examination. All the diagnoses were performed at the Seoul National University Veterinary Medical Teaching Hospital. All frozen serum samples were then transported on dry ice to the laboratory before samples were analyzed.

2.2 | Ecpka elisa

The presence and level of ECPKA in canine sera were then assessed with enzyme-linked immunosorbent assay (ELISA) using anti-canine PKA catalytic subunit Cα (PKA Cα) ELISA kit (Genorise Scientific Inc., Glen Mills, Pennsylvania) following the manufacturer's instructions. Briefly, 100 μL of 4-fold diluted canine serum samples in reagent diluent was added to 96-well ELISA plates precoated with anti-canine PKA Cα antibodies and incubated for 1 hour at room temperature. The plates were further incubated with canine PKA Cα detection antibodies for 1 hour at room temperature, followed by incubation with HRP conjugate for 20 min at room temperature. The plates were then developed with substrate solution for 10 min at room temperature, and the reaction was stopped with 50 μL stop solution. The absorbance was determined at 450 nm with a scanning multiwell spectrophotometer (Molecular Device Co., Sunnyvale, California).

2.3 | Statistical analysis

Graphing and statistical analyses of data were performed using GraphPad Prism software (Systat Software, San Jose, California). Kruskal-Wallis test was used for comparison of serum ECPKA levels among 4 or more different groups. In addition, Mann-Whitney U test non-parametric analysis was performed for analyzing the difference

TABLE 1 Signalment data for participants included in this study

	Cancer	Benign tumour	Non-tumour disease	Healthy
Number	48	18	102	54
Median age (range)	12.0 (0.58-16)	11.5 (2.5-14)	9.0 (0.3-16)	1.6 (0.25-15)
Sex (n)	FS (27), F (3), MC (13), M (5)	FS (6), F (5), MC (6), M (1)	FS (32), F (16), MC (33), M (8), Unknown (13)	FS (8), F (14), MC (10), M (15), Unknown (7)
Breed (*n*)	Shih Tzu (13), Maltese (8), Mogrel (5), C.S. (6), etc (16)	Shih Tzu (3), Maltese (3), M. Poodle (2), Y.T. (1), M.S. (1), C.S. (5), etc (3)	Shih Tzu (16) Maltese (30), M. Poodle (9), Y.T. (9), Mogrel (4), C.S. (8), etc (26)	Shih Tzu (3), Maltese (6), M. Poodle (12) Pomeranian (4), Beagle (15), etc (14)

FS, female spayed; F, female; MC, male castrated; M, male; Y.T., Yorkshire Terrier; M.S., Miniature Schnauzer; C.S., Cocker Spaniel.

TABLE 2 Cancer types and numbers of affected dogs in this study

Cancer type	Number (*n*)
Carcinoma	17
Hematopoietic[1]	13
Sarcoma	8
TCC	6
Hemangiosarcoma	4

Abbreiviation: TCC, transitional cell carcinoma.

[1] Hematopoietic cancers include lymphoma (*n* = 9), acute myeloid leukaemia (*n* = 1), chronic lymphocytic leukaemia (*n* = 1) and mast cell tumour (*n* = 2).

between 2 groups. A *P* value less than 0.05 was determined to be statistically significant.

TABLE 3 Disease types and numbers of dogs with non-tumour diseases in this study

Type of non-tumour disease	Number (*n*)
Gastrointestinal	14
Nerve	12
Dermatologic	12
Cardiovascular	11
Endocrinologic	10
Urinary tract	10
Infectious	9
Hepatobiliary	8
Immune-mediated	6
Orthopaedic	6
Respiratory	3
Hematopoietic	1

3 | RESULTS

We tested sera from 66 dogs diagnosed with tumours (cancers and benign tumours) and 156 non-tumour-bearing dogs (non-tumour diseases and healthy). Signalment data are summarized in Table 1. Male castrated dogs (cancer, *n* = 13; benign tumours, *n* = 6; non-tumour diseases, *n* = 33; healthy, *n* = 10) and female spayed dogs (cancer, *n* = 27; benign tumour, *n* = 6; non-tumour diseases, *n* = 32; healthy, *n* = 8) constituted a larger group than intact male and females, except in healthy dogs. Main breeds in all groups were toy breeds (Shih Tzu, Maltese, Yorkshire Terrier [Y.T], Miniature poodle [M. poodle] and Cocker Spaniel [C.S]), which are the main breeds of indoor dogs in Korea. The types of cancers were categorized as various carcinoma, hematopoietic cancer and sarcoma (Table 2), and benign tumours included adenoma (*n* = 3), sebaceous and splenic hyperplasia (*n* = 4) and lipoma (*n* = 4). The disease types and number of dogs with non-tumour diseases included in this study are summarized in Table 3.

Similar to a previous study in humans,[12] the ELISA assay showed that serum ECPKA level was significantly increased in canine cancer patients compared with non-cancer controls including dogs with benign tumours and non-tumour-bearing controls ($P < .0001$, Kruskal-Wallis test) (Figure 1A). However, serum ECPKA level between dogs with benign tumours and non-tumour controls did not show statistical

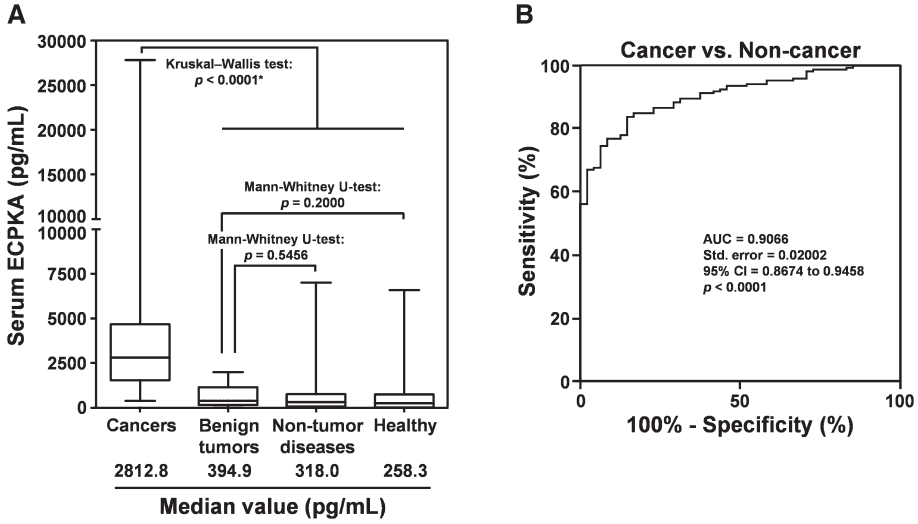

FIGURE 1 Serum extracellular cyclic AMP-dependent protein kinase A (ECPKA) level in dogs with cancers, benign tumours and non-tumour diseases and healthy dogs measured by enzyme-linked immunosorbent assay (ELISA) (A) and receiver operating characteristic (ROC) curve of serum level of ECPKA for the diagnosis of cancer (B). Data are presented as mean ± SEM. Asterisk indicates statistically significant *P* values.

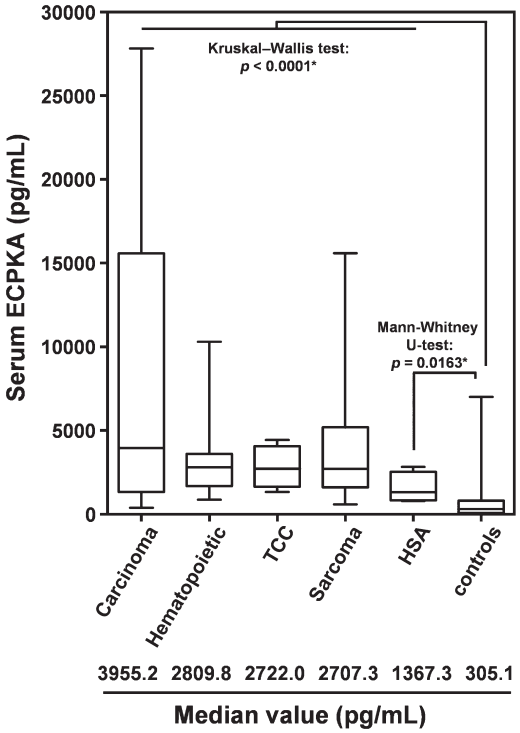

FIGURE 2 Box and whisker plot of serum extracellular cyclic AMP-dependent protein kinase A (ECPKA) level in dogs with different type of cancer and non-cancer controls (benign tumour-bearing, non-tumour disease-bearing and healthy dogs). Data are presented as mean ± SEM. Asterisk indicates statistically significant *P* values.

difference (benign tumours vs non-tumour diseases, *P* = .5456; benign tumours vs healthy, *P* = .2000, Mann-Whitney *U* test). The median value (mean ± SEM) in the cancer group was 2813 pg/mL (5000 ± 942 pg/mL) and ranged from 389 to 27812 pg/mL, which was 7.1-, 8.8- and 10.9-fold higher than benign tumour, non-tumour disease and healthy group, respectively. In addition, serum ECPKA level over 10 000 pg/mL was observed only in dogs with cancer (*n* = 7). The receiver operating characteristic (ROC) curve was also plotted to evaluate the diagnostic value of serum ECPKA as a potential tumour marker as well as to determine the optimal cut-off value to identify the sensitivity and specificity of serum ECPKA in cancer patients, and accuracy was measured by the area under the ROC curve (AUC) (Figure 1B). The AUC of the ECPKA ELISA assay was 0.9066 (95% confidence interval [CI], 0.8674 to 0.9458), and the sensitivity and specificity with a cut-off value of 872.2 pg/mL were 89.58% and 77.01%, respectively.

Serum ECPKA level was significantly increased in all cancer types tested compared with non-cancer controls (*P* < .0001, Kruskal-Wallis test) (Figure 2). Among cancer types, carcinoma showed the most significant increase in serum ECPKA level compared with non-tumour controls (carcinoma, 8276 ± 2326 pg/mL, *n* = 17; transitional cell carcinoma [TCC], 4388 ± 1700 pg/mL, *n* = 6; hematopoietic cancer, 3159 ± 670 pg/mL, *n* = 13; sarcoma, 2817 ± 509 pg/mL, *n* = 8; hemangiosarcoma [HSA], 1557 ± 464 pg/mL, *n* = 4; non-tumour controls, 721 ± 94 pg/mL, *n* = 156). When cancers were grouped as carcinoma (carcinoma and TCC), hematopoietic cancers and sarcoma (sarcoma and HSA), the median, mean and range of serum ECPKA level in each cancer was 3679 pg/mL, 6852 pg/mL (range 389-27 812 pg/mL); 2810 pg/mL, 3159 pg/mL (range 873-10 291 pg/mL); and 2086 pg/mL, 3444 pg/mL (range 589-15 601 pg/mL), respectively. Taken together, these observations indicate that, similar to human cancer patients, dogs with various types of cancer also exhibit increased expression of ECPKA in their sera.

In cancer patients, there was no significant correlation of serum ECPKA level with gender or breed (*P* = .3831 and .5441, respectively, Kruskal-Wallis test) (Figure S1, Supporting information). In addition, although the median age of tumour-bearing dogs was statistically significantly older than that of non-tumour-bearing dogs (*P* < .0001, Kruskal-Wallis test) (Figure S2A), serum ECPKA level did not show statistical difference within cancer patients (*P* = .2486, Mann-Whitney *U* test) or within non-cancer controls depending on age (*P* = .5888, Kruskal-Wallis test) (Figure 2B, C). Furthermore, in dogs with non-tumour diseases, there was no significant difference in serum ECPKA level depending on type of disease (*P* = .6215, Kruskal-Wallis test) (Figure S3). Although ECPKA was detected at high levels in sera from some patients with gastrointestinal or hepatobiliary diseases, serum ECPKA level between cancer patients and dogs with gastrointestinal or hepatobiliary diseases still showed significant statistical difference (*P* = .0003 and *P* < .0001, respectively, Mann-Whitney *U* test). These data further support the notion that increased ECPKA expression in serum is highly cancer-specific.

4 | DISCUSSION

In this study, we demonstrated that serum ECPKA level in dogs with cancers is significantly higher than those in benign tumour-bearing dogs or in dogs with non-tumour controls. All dogs diagnosed with various types of cancer presented significantly increased ECPKA level in their sera compared with those in sera from non-cancer controls, suggesting that serum ECPKA is a potential biomarker for diagnosis of a broad spectrum of canine cancer. In addition, serum level of ECPKA in cancer-bearing dogs as well as noncancer controls did not show any correlation with breed, gender or age. Furthermore, the various noncancerous disease conditions tested in this study did not show any statistically significant increase in serum ECPKA level in dogs. These observations further support the idea that ECPKA is a valuable and specific candidate biomarker for cancer diagnosis in dogs of different breeds or with other disease factors.

Although detection of ECPKA in sera from HSA-bearing dogs using ELISA exhibited a statistically significant increase in serum ECPKA level compared with non-cancer controls (*P* = .0163, Mann-Whitney *U* test), the increase was smallest among the cancer types examined in this study. Further validation with a larger sample size is required to be performed in a large number of HSA serum samples for determining whether this slight increase in serum ECPKA level in HSA patients is attributed to weaker ECPKA expression in this cancer type compared with other types of cancer or the insufficient number of HSA samples (*n* = 4) tested in this study.

Prompt and proper cancer diagnosis is the most important factor for cancer management in human as well as in veterinary medicine. Several biomarkers have been investigated for the early detection of cancer in humans and companion animals including dogs.[1,13,29] One of the most important criteria for a malignancy detection marker is its ability to distinguish cancer from other diseases such as inflammatory and infectious

diseases. However, no single biomarker has been identified to be accurate for diagnosis of malignancy in dogs.[1,13]

In this study, we found that serum ECPKA level was significantly higher in cancer patients than in dogs with various non-neoplastic diseases. In addition, serum ECPKA level in benign tumour-bearing dogs did not exhibit statistical difference from non-tumour controls. These observations suggest that increase in serum ECPKA level is specifically associated with malignancy of tumours but not with other disease conditions. Although this observation needs to be further validated in a larger number of samples and more non-cancerous disorder cohorts, based on our study, we propose that measurement of serum ECPKA level could be an accurate and effective diagnostic tool for screening of various types of canine cancer.

ACKNOWLEDGMENTS

We thank Dr Keri L. Schadler for her help in preparation of this manuscript. This work was supported by a research grant funded by Biattic (S-2016-0517-000) to K.-H.B. and the NRF-2015-Global PhD Fellowship Programme of the National Research Foundation of Korea (NRF) grant funded by the Korean Government (NRF-2015H1A2A1034135) to S.L.

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SUPPORTING INFORMATION

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How to cite this article: Bhang DH, Choi US, Kim BG, et al. Characteristics of extracellular cyclic AMP-dependent protein kinase as a biomarker of cancer in dogs. *Vet Comp Oncol*. 2017;15:1585–1589. https://doi.org/10.1111/vco.12304