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Potential Biomarker of Autoantibody to Extracellular Protein Kinase A in Dogs with Cancer

개의 악성종양 바이오마커로서의 세포외단백질인산화효소 A에 대한 자가항체

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Abstract

Cancer is the leading cause of mortality in adult dogs. Early detection of cancer is needed to prolong the survival rate and quality of life of cancer patients. Cancer biomarker are tools for the early detection of cancer; interest in cancer biomarkers has been increasing due to their safety, convenience, and potential to screen, diagnose, monitor, assess the risk, and predict the prognosis of cancer

patients. In human medicine, various cancer biomarkers have been introduced and used in clinical settings. The extracellular protein kinase A autoantibody (ECPKA-Ab) has been demonstrated as a universal cancer biomarker in humans. However, there have been no studies about ECPKA-Ab in dogs with cancer. Therefore, Serial researches were conducted to evaluate the potency of serum ECPKA-Ab as a cancer biomarker in dogs.

This dissertation comprises three parts. The first part of this study investigated whether high levels of serum ECPKA-Ab and C-reactive protein (CRP) were present in dogs with cancer. Total 487 serum samples were collected from client-owned dogs from the Seoul National University Veterinary Medicine Teaching Hospital and several local animal clinics; sera were collected from 123 healthy dogs, 155 non-neoplastic disease-affected dogs, 42 benign tumor-affected dogs, and 167 malignant tumor-affected dogs. The ECPKA-Ab and CRP levels in the serum samples were analyzed by ELISA method. The neoplastic index (NI) was derived from multivariate regression analysis with ECPKA-Ab and CRP as the independent variables. As a result, both ECPKA-Ab and NI levels were shown to be significantly higher in dogs with malignant tumors than in those without malignant tumors. The area under the receiver operating characteristic was 0.86 and 0.89 for ECPKA-Ab levels and NI value, respectively. The NI value had slightly higher sensitivity, specificity, and accuracy in diagnosing cancer than ECPKA-Ab levels. In conclusion, both ECPKA-Ab and NI value could be used as a universal cancer biomarker in dogs.

The second part of the dissertation tested the dependence of both serum ECPKA and ECPKA-Ab levels on the tumor size. Using the Lewis lung carcinoma

(LCC1) cell line, an *in vitro* study was performed, in which the mRNA levels of PRKAR1B, PRKAR2B and PRKACA according to the seeding cell number were analyzed via qPCR, and the cytosolic/extracellular PKA levels according to the seeding cell number were analyzed via ELISA and Western blotting. An *in vivo* study with C57BL/6J mice was performed to analyze the serum ECPKA and ECPKA-Ab levels according to the tumor size, based on which the mice were divided into five groups (control, tumor100 mm^3, tumor300 mm^3, tumor600 mm^3, and tumor1000 mm^3). Increased cell number induced increased extracellular protein kinase A level in conditioned medium, and increased protein kinase A level in cell, but not affect the mRNA levels. In mouse study, it was demonstrated that the serum ECPKA-Ab levels increased with the increase in tumor size whereas most of mice had undetectable ECPKA levels in their serum. Serum ECPKA-Ab levels increased in proportion to tumor size, and were found to be a more sensitive factor than the antigen.

The third part of the dissertation shows the serial monitoring of serum ECPKA-Ab levels of dogs with naturally occurring lymphoma and transitional cell carcinoma (TCC). The samples were categorized with regards to treatment status as pre-treatment, post-treatment, and relapse; the “post-treatment” samples were further subcategorized into complete remission and progressive disease, according to the therapeutic response. The serum ECPKA-Ab levels were analyzed via ELISA assay. As a result, the serum ECPKA-Ab levels differed significantly with treatment statuses, and the pre-treatment levels differed in response to the CHOP protocol. There was no statistically significant difference in ECPKA-Ab levels when the sera were classified according to location, stage, substage and immunophenotype. All

TCC-affected dogs treated with piroxicam had progressive disease and their serum ECPKA-Ab levels increased with the progression of the cancer. The serum ECPKA-Ab levels could be a predictive cancer biomarker for lymphoma and TCC in dogs.

In conclusion, the serum ECPKA-Ab levels could be a universal cancer biomarker in dogs; it could provide several details about the presence of various cancer, the prediction of therapeutic response to specific chemotherapeutic drugs to certain cancer, and monitoring the progression status of some cancers.

Keywords: autoantibody / biomarker / cancer / dog / extracellular protein kinase A
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ABBREVIATIONS

Ab	Antibody
AFP	Alpha-fetoprotein
ANOVA	Analysis of variance
ASAC	Anal sac adenocarcinoma
AUROC	Area under the curve of receiver operating characteristic
BMD	Bernese mountain dog
BTA	Bladder tumor antigen
CA	Cancer antigen
Car	Carcinoma
CEA	Carcinoembryonic antigen
CM	Castrated male
CR	Complete remission
CRC	Colorectal carcinoma
CRP	C-reactive protein
CTC	Circulating tumor cells
CV	Cardiovascular disease
Der	Dermatologic disease
DHS	Disseminated histiocytic sarcoma
DPBS	Dulbecco's phosphate buffered saline
ECPKA	Extracellular protein kinase A

EGFR1	Epidermal growth factor receptor 1
EIA	Enzyme-immunoassay
ELISA	Enzyme linked immunosorbent assay
Endo	Endocrinic disease
ER	Estrogen receptor
F	Female
FBS	Fetal bovine serum
FISH	Fluorescent in situ hybridization
FP	False positive
GI	Gastrointestinal disease
GIST	Gastrointestinal stromal tumor
HSA	Hemangiosarcoma
HB	Hepatobiliary disease
HCC	Hepatocellular carcinoma
hCG	Human chorionic gonadotropin
Hema	Hematopoietic/lymphoid cancer
HER	Human epidermal growth factor receptor
HG-DMEM	High glucose Dulbecco's modified Eagle's medium
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
Immune	Immune-mediated disease
Inf	Infectious disease
IP	Intraperitoneal

LDH	Lactate dehydrogenase
LLC1	Lewis lung carcinoma
Lym	Lymphoma
M	Male
MCA	Methylocholanthrene
Mel	Malignant melanoma
MMGT	Malignant mammary gland tumor
MMP	Metalloproteinase
MST	Mean survival time
Neu	Neurologic disease
NI	Neoplastic index
NPV	Negative predictive value
NSCLC	Non-small cell lung cancer
OS	Organ system
OS	Overall survival
PAC	Pulmonary adenocarcinoma
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Progressive disease
PFS	Progression-free survival
PKA	Protein kinase A
PP	Pathophysiologic process
PPV	Positive predictive value

PR	Progesterone receptor
PR	Partial remission
PSA	Prostate specific antigen
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
RCC	Renal cell carcinoma
RECIST	Response evaluation criteria in solid tumors
Res	Respiratory disease
ROC	Receiver operating characteristic
SA	Sequence analysis
Sar	Sarcoma
SC	Subcutaneous
SCC	Squamous cell carcinoma
SD	Static disease
SF	Spayed female
SM	Skeletomuscular disease
TBST	Tris-buffered saline-Tween20
TCC	Transitional cell carcinoma
TK1	Thymidine kinase 1
Uro	Urologic disease
VEGF	Vascular endothelial growth factor
WHO	World health organization

APPENDIX Publications arising from this thesis

Ryu MO, Kim BK, Choi US, Baek KH, Song YK, Li Q, Seo KW, Ryeom S, Youn HW, and Bhang DH. 2018. Extracellular cyclic AMP-dependent protein kinase autoantibodies and C-reactive protein as serum biomarkers for diagnosis of cancer in dogs. *Veterinary and Comparative Oncology*.

General Introduction

The disease occurring highest mortality in dogs is cancer (Fleming et al., 2011). The cancer incidence increases as dogs age (Baioni et al., 2017, Merlo et al., 2008). Like as human, early diagnosis of cancer increases the kinds of cancer treatment and also increases cure rates and survival rate (Parkin et al., 2005, Wardle et al., 2015). Diagnostic tools for cancer have been developed and, therefore, incidence rate of cancer seems to be increased (Grüntzig et al., 2015). Heretofore, most useful diagnostic tool for cancer included radiography, ultrasonography, computed tomography, magnetic resonance imaging, cytology, histopathology, and polymerase chain reaction. However, they have respective limitations including low sensitivity or specificity to diagnose malignancy, requiring anesthesia, requiring expensive equipment, or limitations to access tumors due to invasiveness.

Cancer biomarker is a biological molecule that is produced by tumor cell or associated with existing cancer in the body (Füzéry et al., 2013, Duffy, 2013). Simultaneously, cancer biomarkers can inform presence or prognosis of cancer. Potential clinical application of cancer biomarkers includes risk assessment before suffering cancer, diagnosis of cancer, prediction of prognosis, prediction of therapeutic response to specific anticancer drug, monitoring therapeutic response, and monitoring reoccurrence (La Thangue and Kerr, 2011, Buyse et al., 2011, Duffy and Crown, 2008, Febbo et al., 2011). In human medicine, many cancer biomarkers have been studied and developed for commercialization (Mehta et al., 2010). Biomarkers used in clinics for diagnosis specific cancer include prostate specific

antigen for prostate adenocarcinoma, α-fetoprotein (AFP) for liver cancer, and cancer antigen 125 (CA125) for ovarian cancer (Mordente et al., 2015, Salman et al, 2015). Prognostic cancer biomarkers which inform survival rates of the patient includes BRCA1, CEA, c-kit, and circulating tumor cells (Mehta et al., 2010). Predictive biomarkers which predict therapeutic respond to specific anticancer drug include estrogen receptor, progesterone receptor, EGFR1, BRCA1 and K-ras (Mehta et al., 2010). Unlike the biomarkers mentioned earlier, universal cancer biomarker is a molecule indicating that the cancer is in the body, regardless of the type of cancer (Nesterova et al., 2006a, Nesterova et al., 2006b, Essaghir and Demoulin, 2012). Universal cancer biomarkers provide information that there may be cancer somewhere in the body, allowing further diagnosis to diagnose cancer.

Extracellular cyclic AMP-dependent protein kinase A (ECPKA) was found in 2000 by Cho ES et al (Cho et al., 2000b). Cancer cells excrete protein kinase A out of cells whereas Non-cancer cells do not (Cho et al., 2000b). It is demonstrated that ECPKA could be a universal cancer biomarker in human medicine by demonstrating that ECPKA existed higher level in patients with various cancer than healthy people (Cho et al., 2000b, Wang et al., 2007, Kita et al., 2004). Another study conducted with dogs showed that ECPKA concentrations was significantly increased in serum of dogs suffering cancer than healthy dogs (Bhang et al., 2017). However, ECPKA was nonstable and activity was decreased with freezing/thawing (Cho et al., 2000a). The presence of autoantibodies against various cancer biomarkers were demonstrated after then (Nesterova et al., 2006a). ECPKA autoantibody (ECPKA-Ab) was also determined in serum of human patient. Similar with other autoantibodies to cancer biomarkers including AFP, CA125, and vascular

endothelial growth factor, ECPKA-Ab had higher sensitivity, specificity than ECPKA on cancer diagnosis (Nesterova et al., 2006a). ECPKA-Ab also increased in serum of patients with various cancer, therefore, it could be used as universal cancer biomarker in human (Nesterova et al., 2006b).

In veterinary medicine, a few cancer biomarkers have been studied. Moreover, there is very few cancer biomarkers commercially used. However, the need to cancer biomarkers is enlarged because cancer biomarkers help diagnosis of cancer earlier and easier. Samples for detecting cancer biomarkers are easily obtained, such as blood or urine (Kulasingam and Diamandis, 2008). The objective of this study is to evaluate whether ECPKA-Ab is detectable in canine serum and to evaluate whether ECPKA-Ab is increased in dogs suffering cancer like as human, and to search potency of ECPKA-Ab in clinical application in dogs. In this study, I collected serum samples of client-owned dogs with various disease and determined concentrations of ECPKA-Ab in serum via immunosorbent assay for demonstrating the hypothesis that ECPKA-Ab could be a universal cancer biomarker in dogs with variable applications.

LITERATURE REVIEW

1. Introduction

Cancer is a very lethal disease in dogs, and research on the diagnosis and treatment of cancer continues to be of interest as it is in human medicine. As examination method has been developed, annual tumor incidence rate has been increased (Grüntzig et al., 2015). There are many researches about cancer incidence in dogs and it is summarized in Table L.1. According to a recent study conducted in Italy from 2001 to 2008 (Baioni et al., 2017), there were 804 malignant tumors per 100,000 dogs and 897 benign tumors per 100,000 dogs. Another study conducted in North America from 1984 to 2004 (Fleming et al., 2011) also showed that frequency of neoplastic disease increases with age and that cancer is the leading cause of death in adult dogs (>1 year), while the leading cause of death in puppy (<1 year) is infectious disease (Fig L.1).

The incidence of tumors was higher in females than in males, and the incidence of tumors was increased with age regardless of gender (Fleming et al., 2011, Baioni et al., 2017). A study conducted in Italy with tumor biopsy specimens showed that cancer incidence in female dogs was three times higher than in male dogs due to high rates of mammary cancer (Merlo et al., 2008). A study conducted in Swiss Canine Cancer Registry from 1955 to 2008 showed that influence of sex on tumor incidence depend on the examination method (Grüntzig et al., 2016). When performing analysis grouped cancer based on cell origin, cancer incidence is

higher in female on adenoma/adenocarcinoma, but cancer incidence of hemangiosarcoma or squamous cell carcinoma was lower in female than male (Grüntzig et al., 2016). In females, the main tumor site is the mammary gland, the skin, and the ovary, while the main tumor site of the male is the skin, testis, and spleen (Baioni et al., 2017).

Biomarkers are defined as "a cellular, biochemical, and/or molecular (including genetic and epigenetic) characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention by The Biomarkers Definitions Working Group of the National Institutes of Health (BiomarkersDefinitionsWorkingGroup, 2001). In particular, cancer biomarkers are defined as "biomarkers produced by tumor cell or associated with cancer producing mechanism" (Füzéry et al., 2013, Duffy, 2013). Cancer biomarkers could be classified by biochemical characteristic as DNA, RNA, proteins, peptides, hormones, metabolites, exosome, and circulating tumor cells (Mordente et al., 2015) and could be classified by functional characteristic into diagnostic marker, predictive marker, prognostic marker, pharmacodynamics marker, marker for therapeutic monitoring, and marker for risk assessment (La Thangue and Kerr, 2011, Buyse et al., 2011, Duffy and Crown, 2008, Febbo et al., 2011). Potential clinical application of cancer biomarker is showed in Figure L.2 (Mehta et al., 2010).

In an article dealing with global statistics for human cancer, early detection program could increase incidence rate of cancer but also increase survival time of cancer patients (Parkin et al., 2005, Wardle et al., 2015). This is why researchers have studied for developing early cancer detection program. To date, lots of study

have performed about cancer biomarkers in human medicine (Mehta et al., 2010, Cho, 2007). S Metha et al reviewed about cancer biomarkers of human medicine (Mehta et al., 2010). The authors introduced lots of prognostic markers including BRCA1, carcinoembryonic antigen (CEA), c-kit, circulating tumor cells (CTC), estrogen receptor (ER) and predictive markers including c-kit, epidermal growth factor receptor 1 (EGFR1), K-ras and their clinical significance. Table L.2 shows cancer biomarkers used in clinical practice in human medicine. Developing cancer biomarkers is considered to tool of early detection of cancer and to give public health to economical advantage (Baron, 2012, Srivastava and Gopal-Srivastava, 2002). Although there is a drawback to overdiagnosis in false positive patients because the accuracy and sensitivity are not one hundred percentages, the cancer biomarker has the merit of being able to conveniently examine by safe method, and moreover, it could increase the diagnostic yield in specific group by combining with other panels (Baron, 2012).

2. Canine cancer biomarker

2.1. Mammary gland tumor

As mentioned earlier, because mammary tumors are the most common tumors in female dogs and malignancies are very important issues, there is a strong interest in biomarkers for malignant mammary gland tumors (Merlo et al., 2008, Dorn et al., 1968, Grüntzig et al., 2015). Many breast cancer biomarkers in humans have been found, including cancer antigen 15.3 (CA 15.3), carcinoembryonic

antigen (CEA), estrogen receptors, progesterone receptors, human epidermal growth factor receptor 2 (HER2), and some of them have been used clinically (Gutman and Kessler, 2006, Mehta et al., 2010, Duffy et al., 2010, Singhai et al., 2011, Ross et al., 2003, Samy et al., 2010, Hammond et al., 2010). In one study, an experiment was conducted to determine whether human breast cancer biomarkers could be used as mammary gland tumor biomarkers in dogs (Campos et al., 2012). As a result, CEA did not have significant meaning, and lactate dehydrogenase (LDH) and CA15.3 were found to increase in serum when metastasis was present. In particular, it was concluded that CA15.3 could distinguish metastasis from non-metastasis. In another study, nitric oxide and sialic acid levels in serum of dogs with malignant mammary gland tumors were significantly higher than those of healthy dogs (Tascene et al., 2012). In the other study, immunological biomarkers in dogs with malignant mammary gland tumors were evaluated and assessed whether these biomarkers were associated with metastasis or histologic characteristics (Estrela-Lima et al., 2016). A total of 10 chemokines/cytokines (CXCL 10, IL-2, IL-4, IL-6, CXCL-8, IL-10, IL-15, IL-18, IFN-r, and TNF-a) were analyzed in dogs with healthy and mammary cancer. As a result, plasma IL-10 was found to be associated with survival rate. When plasma IL-10 levels > 15 pg/mL, survival times were significantly lower than those ≤ 15 pg/mL (Area under the curve of Receiver Operating Characteristic curve = 0.84).

There are several studies for discovering biomarkers about mammary gland tumor using *in vitro* studies. A study investigating microenvironment of inflammatory mammary carcinoma in dogs using patients' tissue samples revealed that gene expression level was higher in inflammatory mammary carcinoma on

cyclooxygenase 2 (p=0.0004), synuclein gamma (p=0.0006), tribbles 1 (p=0.025), vascular endothelial growth factor (p=0.017), and receptor of macrophage colony stimulating factor 1 (p=0.045) than non-inflammatory carcinoma (Raposo et al., 2017). The authors suggest that upstreaming and downstreaming of SNCG and tribbles 1 need to be further studied considering metastasis is linked to factors in the tumor microenvironment. Some researchers were tried to identify extracellular vesicles of mammary gland tumor of dogs and cats and succeed to obtain the extracellular vesicles (Sammarco et al., 2018).

2.2. Lymphoma

Lymphoma is the most common hematopoietic cancer of dogs, which accounts for 2% to 24% of all cancers in dogs (Dorn et al., 1968, Kaiser, 1981, Zandvliet, 2016, Grüntzig et al., 2016, Merlo et al., 2008). In addition, canine lymphoma has a good response to chemotherapy, and there is a significant difference in survival rate when treated and when not treated (Withrow et al., 2013, Zandvliet, 2016). Therefore, in canine lymphoma, biomarker for prognosis as well as biomarker for presence of lymphoma has been studied.

Several studies suggest to vascular endothelial growth factor (VEGF) and metalloproteinase (MMP) 9 as biomarkers for canine lymphoma (Aresu et al., 2014, Schaefer et al., 2007). VEGF and MMPs are producted during cacerogenesis via transforming growth factor beta (Bauvois, 2012, Ten Dijke and Arthur, 2007, Bergers et al., 2000, Aresu et al., 2014) and they are human lymphoma's useful biomarkers (Citak et al., 2008, Hazar et al., 2004, Bauvois, 2012). Aresu et al

showed that lymphoma dogs have higher act-MMP-9 ($p<0.01$) and VEGF ($p<0.05$) levels in their plasma, especially in T-cell lymphoma (Aresu et al., 2014). Furthermore, act-MMP-9 and VEGF levels were decreased during chemotherapy in B-cell lymphoma dogs ($p<0.01$) and it suggests prognostic roles in lymphoma dogs.

A study has shown that quantification of plasmid DNA can be used to assess the prognosis of canine lymphoid neoplasia (Schaefer et al., 2007). Similar to the increase in cell-free circulating DNA in plasma of human cancer patients, plasma DNA was found to be significantly higher in dogs with lymphoma or lymphoid leukemia ($p<0.0001$). In addition, plasmid DNA > 25 ng/mL in lymphoid neoplasia was found to have a shorter remission time than in the < 25 ng/mL dogs.

For lymphoma biomarker, thymidine kinase 1 (TK 1) activity has been most studied (Elliott et al., 2013, von Euler et al., 2004, Von Euler et al., 2009, Sharif et al., 2012, NAKAMURA et al., 1997). Most of the studies suggests that TK1 activity in serum or plasma of lymphoma dogs is significantly higher than healthy dogs and after chemotherapy, TK1 activity decreased with the reduction of the tumor mass and increased prior to relapse (NAKAMURA et al., 1997, Sharif et al., 2012, von Euler et al., 2004). In contrast to the other study, JW Elliott et al. showed that TK1 was normal in 53% of total lymphoma dogs at the time of diagnosis and that TK1 levels were not correlated to stage, duration of first remission, and survival time (Elliott et al., 2013).

Serum C-reactive protein (CRP) was useful factor to determine remission status (Nielsen et al., 2007). CRP levels were significantly different between complete remission, partial remission, progressive disease, and stable disease in

dogs with lymphoma after chemotherapy and with median values of 1.91, 2.48, 1.77, 8.7 mg/L, respectively. Similarly, I Alexandrakis et al. developed canine lymphoma blood test (cLBT) utilizing haptoglobin and C-reactive protein as a serum biomarker to monitor therapeutic response in canine lymphoma (Alexandrakis et al., 2017). cLBT score correlated with survival time and parallel to recurrence and remission of lymphoma.

2.3. Universal cancer marker

Universal cancer markers are not markers that diagnose specific tumors like lymphoma or mammary gland tumor, but markers that can distinguish cancer patients from healthy individuals (Nesterova et al., 2006a, Nesterova et al., 2006b, Essaghir and Demoulin, 2012). In human medicine, telomerase activity, extracellular cAMP dependent protein kinase A (ECPKA), follicle stimulating hormone, cytochrome P450, autoantibodies of leukocyte antigen F were discovered as a biomarker for various cancer types so far (Essaghir and Demoulin, 2012).

In veterinary medicine, TK1 activity and ECPKA were discovered in dogs of various cancer types. KA Selting et al. showed that TK1 activity could be a screening biomarker for occult cancer dogs and it is higher in all cancer especially lymphoma and histiocytic sarcoma (Selting et al., 2015b, Selting et al., 2016b). The authors also showed that neoplastic index made by a combination of TK1 activity and CRP level has higher accuracy than TK1 activity only for diagnosis of cancer. Recent study showed that serum ECPKA concentration is higher in cancer dogs than healthy and non-neoplastic diseased dogs (Bhang et al., 2017). There is a study

about many cancer dogs have detectable VEGF concentration in their blood and lower endostatin level than healthy dogs (Troy et al., 2006).

2.4. Others

Research on various biomarker associated with a variety of cancer have been conducted in veterinary medicine. Serum collagen XXVII and TK1 activity have been studied as biomarkers for hemangiosarcoma. Serum collagen XXVII peptide concentration in dogs with large metastatic hemangiosarcoma was 9.5 fold higher than healthy dogs and area under the receiver operating characteristic curves for canine hemangiosarcoma was 0.83 (Kirby et al., 2011). The research showed that serum collagen XXVII peptide concentration was decreased after tumor removal and chemotherapy, while increased with recurrence. A prospective study determined serum TK1 activity of dogs with hemoabdomen showed that serum TK1 activity can help to distinguish the hemangiosarcoma versus benign diseases (Thamm et al., 2012). Cathepsin K, a lysosomal protease secreted by osteoclasts, has been shown to be elevated in the serum of osteosarcoma dogs and has been shown to gradually decrease after treatment with palliative radiation, doxorubicin, and pamidronate (Schmit et al., 2012). In a study conducted to find early diagnostic markers or screening markers for disseminated histiocytic sarcoma (DHS) in Bernese Mountain Dogs (BMD), a total of eight factors were found to differ between DHS dog and normal dog, and finally serum ferritin was found to be higher in early DHS dogs (Nielsen et al., 2012). The authors suggested performing a DHS screening program every 6 months in BMD over 4 years of age, which included

measurement of serum ferritin concentration with radiological diagnosis. Serum vitamin D level was also found to be related to cancer risk. A study conducted with Labrador retrievers, vitamin D level was lower in dogs with mast cell tumor (Wakshlag et al., 2011) and another study conducted with hemoabdomen dogs, vitamin D level was lower in neoplastic group than non-neoplastic group (Selting et al., 2016a).

A biomarker assay using urine as a sample as well as blood was also studied. A recent study has shown that urinary and prostatic carcinomas can be diagnosed by detecting BRAF V595E mutation in urine (Mochizuki et al., 2015).

3. Extracellular protein kinase A

3.1. Characteristics of protein kinase A

A prominent member of the AGC kinase which is regulating various cellular processes including cell division and glucose metabolism is the cAMP-dependent protein kinase "protein kinase A (PKA)" (Taylor et al., 2012). PKA has an essential role of regulating many cell signaling as a serine/threonine kinase (Sim and Scott, 1999, TASKÉN and AANDAHL, 2004). Molecular component of PKA is composed of catalytic 'C' subunit and regulatory 'R' subunit (Corbin et al., 1977, Corbin et al., 1978, Zoller et al., 1979, Bechtel et al., 1977, Ringheim and Taylor, 1990). The 'R' subunit exist in two type with two isoforms of each type which was determined via cDNA cloning; RIα, RIβ, RIIα, and RIIβ (Lee et al., 1983, Clegg et al., 1988, Øyen et al., 1989, Jahnsen et al., 1986, Skalhegg and Tasken, 2000).

Inactive PKA holoenzyme comprised of two R subunit and two C subunit and PKA type is classified into PKAI and PKAII which contain RI or RII, respectively (Rannels and Corbin, 1980, Taylor et al., 2012). According to regulatory subunit type, PKA tetramer localize other sites in cellular space and expression frequency are different according to cell type (Taylor et al., 2012). Catalytic 'C' subunit have three isoforms (Cα, Cβ, and Cγ) and predominant isoform is Cα (Uhler et al., 1986, Beebe et al., 1990). When cAMPs bind each R subunit, R subunits undergo a conformational change resulting in releasing active C subunits to downstream cascade (Skalhegg and Tasken, 2000, Turnham and Scott, 2016). Without cAMP, the R subunit suppress the activity by binding C subunit (Taylor et al., 2012). PKA signaling system is briefly shown in Figure L.3.

3.2. Discovery of extracellular protein kinase A in cancer

For the first time in 2000, it has been reported that PKA is excreted out of cells in cancer cells by ES Cho et al, which is called extracellular protein kinase A (PKA) (Cho et al., 2000b). They determined that ECPKA is Cα unit which is active form of PKA via western blot analysis. In the article, they showed that expression of regulatory subunits in cells was related to expression level of ECPKA. ECPKA was overexpressed in case of RIα subunit overexpression whereas downregulated in case of RIIβ subunit overexpression. The study also showed that excretion of PKA out of cells required NH2-terminal myristyl group of catalytic subunit. In addition, the researchers found the ECPKA was highly elevated in the serum of cancer patients including renal cell carcinoma, melanoma, lymphoma than normal

people ($p<0.05$, one-way ANOVA). The authors published another article about ECPKA (Cho et al., 2000a). They showed that ECPKA was sensitive to temperature than intracellular protein kinase A, so only 20% of activity was remnant after 2 cycles of freezing/thawing. Another study conducted in China, researchers compared ECPKA levels in 374 normal people versus 229 cancer patients (Wang et al., 2007). Significantly higher levels of ECPKA were found in cancer patient and greater than 70 percentage of normal people had very low ECPKA level in their serum. A study about ECPKA levels in melanoma patients revealed that surgical removal of tumor made ECPKA levels to decrease (Kita et al., 2004).

Recently, serum ECPKA level was evaluated in dogs with cancer (DH Bhang, Vet Comp Oncol, 2016). In the study, total 48 dogs with cancer was compared to benign tumor (n = 18), non-neoplastic disease (n = 102), and healthy (n = 54) and as a result, it revealed higher ECPKA in cancer dogs than other dogs ($p<0.0001$, Kruskal Wallis test). Receiver operating characteristic curves resulted in 0.9066 of area under the curve, 77.01% of specificity, and 89.58%% of sensitivity with a cut-off 877.2 pg/ml.

3.3. Autoantibody of extracellular protein kinase A in human cancer

Studies have shown that ECPKA as well as ECPKA-Ab are highly elevated in the serum of cancer patients in the mid-2000s. M Nesterova et al presented that an ECPKA autoantibody detection method via immunoassay and they confirmed high titer of ECPKA autoantibody in serum of patients with various cancer including carcinomas, sarcomas, melanoma and thymoma (Nesterova et al., 2006a).

In the study, the receiver operating characteristic plots showed that ECPKA autoantibody detection method have better sensitivity and specificity than ECPKA antigen detection method (90% sensitivity and 88% specificity in autoantibody assay; 83% sensitivity and 80% specificity in antigen assay) (Nesterova et al., 2006a). In addition, they have demonstrated that autoantibody detection method is useful by detecting autoantibodies of well-known cancer biomarkers including prostate specific antigen (PSA), alpha-fetoprotein (AFP), CA125, VEGF at high concentrations in serum of cancer patients (shown in Figure L.4) (Nesterova et al., 2006b). W Loilome et al reported that increased ECPKA autoantibody levels of patients suffering liver fluke-associated cholangiocarcinoma is associated with switching of the PKA regulatory subunit (Loilome et al., 2012). They demonstrated that cholangiocyte proliferation and transformation might be induced by change of R isoform from PRKAR2B/PKAII to PRKAR1A/PKAI. This is similar result with previously mentioned article about increased ECPKA levels associated with regulatory subunit (Cho et al., 2000a).

There was a report about change titer of ECPKA-Ab in respond to treatment in Non-Hodgkin's lymphoma (Choi et al., 2006). In the report, ECPKA-Ab was increased in serum of Non-Hodgkin's lymphoma patient and the titer decreases with response to treatment (complete or partial remission) and recurred patient had increased titer of ECPKA-Ab.

Table L.1. Tumor incidence rate (all sites) estimated in pet dogs by country and tumor characteristics.

Country	Years	Canine population		Tumors	Incidence Rate[b]	Reference
		Base Population	No.[a]			
UK	1997-1998	UK insured dogs[c]	130,684	Cancers[d]	747.9	(Dobson et al., 2002)
				All neoplasia related claims[d]	1,948	
California USA	1963-1966	Alameda Country	1,031	Cancers	381	(Dorn et al., 1968)
				Nonmalignant tumors	1,130	
Ontario	1999	Veterinary Clinics	63,500	Cancers	850	(Reid-Smith et al., 2000)
Canada				All tumors	3,970	
Italy	1985-1994	Genoa County	127,600	Cancers	310	(Merlo et al., 1995)
				Nonmalignant tumors	760	
Italy	2001	Local Health Unit	9,182	Cancers	958.4	

[a]Size of the estimated canine population at risk. [b]Crude rate defined as the number of cases per 100,000 dogs/year.

[c]All dogs insured with a single UK pet insurance company. [d]Claims for veterinary treatment identified as being related to neoplasia.

(adopted by DF Merlo et al, JVIM 2008, Table 1)

Table L.2. Cancer biomarkers clinically used in human medicine.

Prognostic biomarker	Type of cancer		Detection	Reference
BRCA1	Breast	high expression of BRCA1 confers worse prognosis in untreated patients	IHC	(James et al., 2007)
CEA	CRC	Elevated preoperative CEA levels in resectable colorectal cancer is associated with poor prognosis	IHC	(Wolmark et al., 1984, Wanebo et al., 1978)
c-kit	GIST	GIST patients have a better prognosis if they harbor a mutation in exon 11 of the c-KIT gene	pathway detection via FDG-PET	(Singer et al., 2002)
ColoPrint CTC	CRC	prognosis for colorectal cancer patients	microarray	(Glas et al., 2009)
	CRC	colorectal patients with ≥3 CTC/7.5 ml of peripheral blood were associated with shorter PFS and OS, i.e. poor prognosis	CTC	(Cohen et al., 2009)
	Breast	breast cancer patients with ≥5 CTC/7.5ml of peripheral blood are associated with shorter PFS and OS, i.e. poor prognosis	CTC	(Cristofanilli et al., 2004)
	Prostate	≥5 CTC/7.5ml of peripheral blood is associated with poor prognosis	CTC	(Cristofanilli et al., 2004)
ER	Breast	patients with ER-positive breast tumors have better survival than patients with hormonal negative tumors	IHC	(Early breast cancer trialists' collaborative group, 1998)
eXageneBC	Breast	provides prognosis in node-positive or node-negative breast cancer patients	FISH	(Davis et al., 2007)
Her2/neu	Breast	patients with Her2/neu-positive breast tumors are more aggressive and have a worse prognosis compared to Her2/neu-negative tumors	FISH	(Mass et al., 2005)
K-ras	NSCLC	K-ras mutation is associated with poor prognosis in NSCLC patients	SA	(Zhu et al., 2008)

MammoPrint	Breast	A 70-gene prognostic assay used to identify breast cancer cases at the extreme end of the spectrum of disease outcome by identifying patients with good or very poor prognosis	microarray	(Van't Veer et al., 2002)
Mammostrat®	Breast	This standard purely prognostic test uses five antibodies with manual slide scoring to divide cases of ER-positive, lympho node negative breast cancer tumors treated with tamoxifen alone into low-, moderate- or high-risk groups	IHC	(Ring et al., 2006)
Oncotype DX	Breast	A 21-gene multiplex test used for prognosis to determine 10-year disease recurrence for ER-positive, lympho node negative breast cancers using a continuous variable algorithm and assigning a tripartite recurrence score	qRT-PCR	(Goldstein et al., 2008, Paik et al., 2004)
PR	Breast	Patients with PR-positive breast tumors have better survival than patients with hormonal-negative tumors	IHC	(Dowsett et al., 2006)
VEGF	RCC	overexpression of VEGF is associated with poor prognosis in clear cell renal carcinoma patients	IHC	(Oldenhuis et al., 2008)
Predictive biomarker	Type of cancer		Detection	Reference
BRCA1	Breast	high expression of BRCA1 in breast cancer can predict response to chemotherapy	IHC	(James et al., 2007)
c-kit	GIST	GIST patients carrying the mutation on exon 11 of the c-KIT gene benefit from imatinib and sunitinib treatment, however most patients develop resistance to these over time	SA	(DeMatteo et al., 2009)
EGFR1	NSCLC	EGFR1 mutations in patients with NSCLC are predictive for response to either gefitinib or erlotinib treatment	IHC	(Sequist et al., 2007)
	CRC	EGFR1 gene am;lification appears to be a predictive factor for response to anti-EGFR1 antibody treatment in CRC	PCR	(Amado et al., 2008)
ER	Breast	high cellular expression of ER predicts benefit from tamoxifen-based chemotherapy	IHC	(Early breast cancer trialists'

				collaborative group, 1998, Paik et al., 2006)
Her2/neu	Breast	breast cancer patients with Her2/neu overexpressing tumors benefit from treatment with trastuzumab in the metastatic as well as in the adjuvant setting	FISH	(Mass et al., 2005)
K-ras	NSCLC	K-ras mutation positivity in NSCLC patients predicts lack of benefit from adjuvant chemotherapy in early disease and resistance to treatment with EGFR TKI in advanced disease	SA	(Mascaux et al., 2005)
	CRC	K-ras mutation positivity in stage Ⅳ CRC patients predicts considerably less benefit from EGFR-specific antibody like cetuximab and panitumumab	PCR	(Amado et al., 2008)
NuvoSelect	Breast	a combination of several pharmacogenomic genesets used primarily to guide selection of therapy in breast cancer patients. This test also provides the ER and HER2 mRNA status	microarray	(Rouzier et al., 2005, Ayers et al., 2004)
PR	Breast	high cellular expression of PR predicts benefit form tamoxifen-based chemotherapy	IHC	(Oldenhuis et al., 2008, Dowsett et al., 2006, Elledge et al., 2000)
Roche AmpliChip	Breast	low expression of CYP2D5 predicts resistance to tamoxifen-based chemotherapy in breast cancer patients	microarray	(Hoskins et al., 2009)
Rotterdam Signature	Breast	A 76-gene assay used to predict recurrence in ER-positive breast cancer patients treated with tamoxifen	microarray	(Wang et al., 2005)

Abbreviation; CRC, colorectal tumor; CTC, circulating tumor cells; EGFR, epidermal growth factor receptor; ER, estrogen receptor; FISH, fluorescent in situ hybridization; GIST, gastrointestinal stromal tumor; IHC, immunohistochemistry; NSCLC,

non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; PR, progesterone receptor; qRT-PRC, quantitative real time polymerase chain reaction; RCC, renal cell carcinoma; SA, sequence analysis; VEGF, vascular endothelial growth factor

(Adopted by Mehta S et al., 2010, Therapeutic advances in medical oncology, Table 1 and 2)

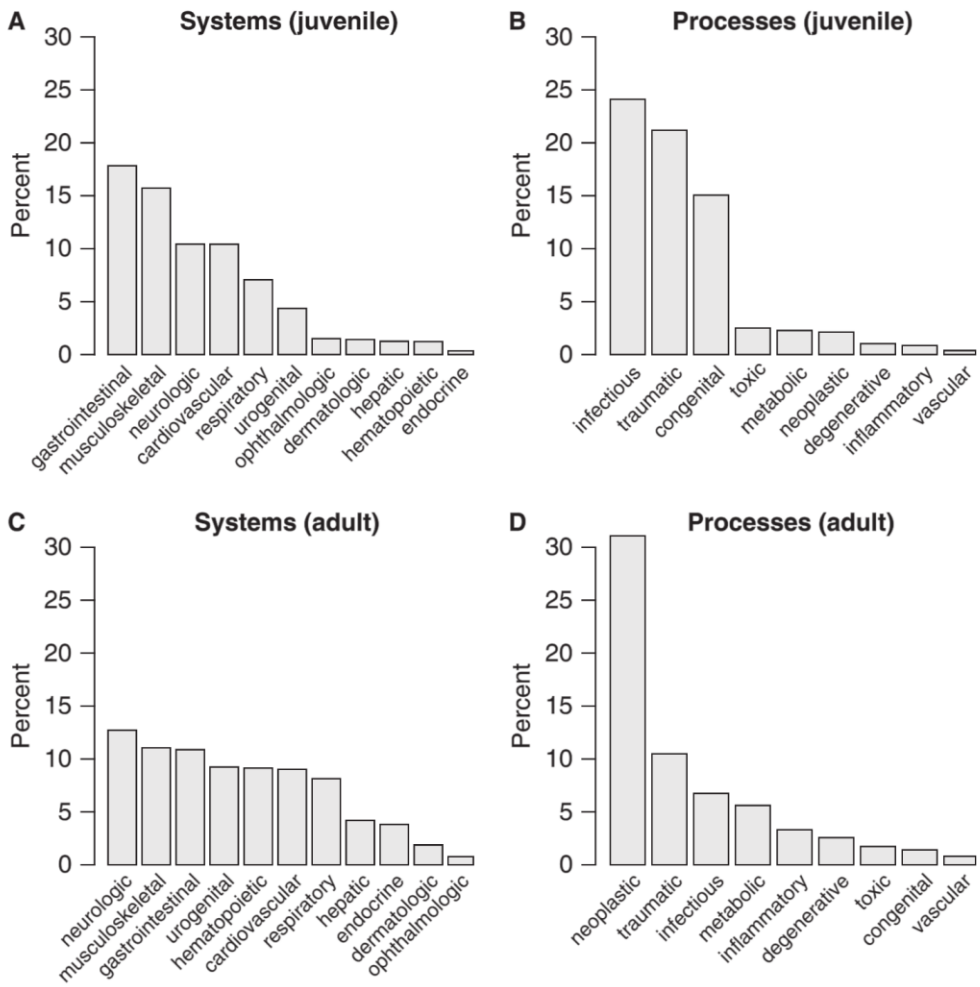

Figure L.1. Proportion of deaths attributable to each organ system (OS) and pathophysiologic process (PP) category for juvenile and adult dogs.

Among 9,859 juvenile dogs (up to 1 year), 2,792 were unclassified for OS (A, n = 7,067) and 3,004 were unclassified for PP (B, n = 6,855). Among 64,697 adult dogs (1 year or greater), 12,374 individuals were unclassified for OS (C, n = 52,323) and 23,438 individuals were unclassified for PP (D, n = 41,259)

(Adopted by JM Fleming et al, 2011, JVIM)

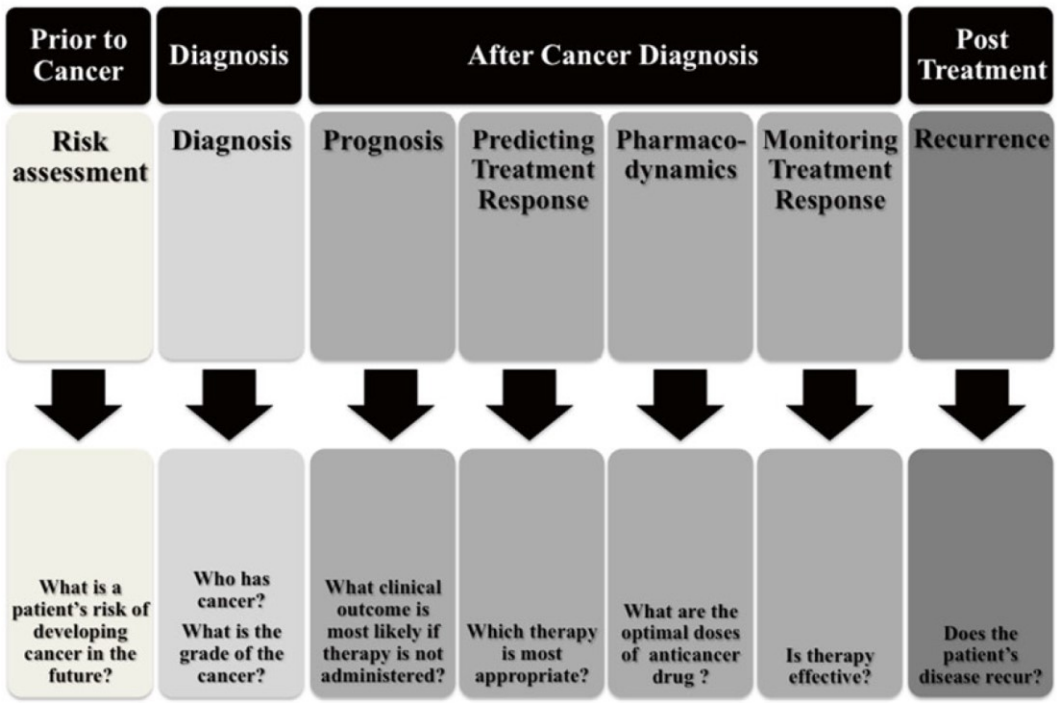

Figure L.2. Potential fields of application of a cancer biomarker test.

Adopted by Mordente A et al, 2015, Advances in Cancer Biomarkers, Figure 2.1.

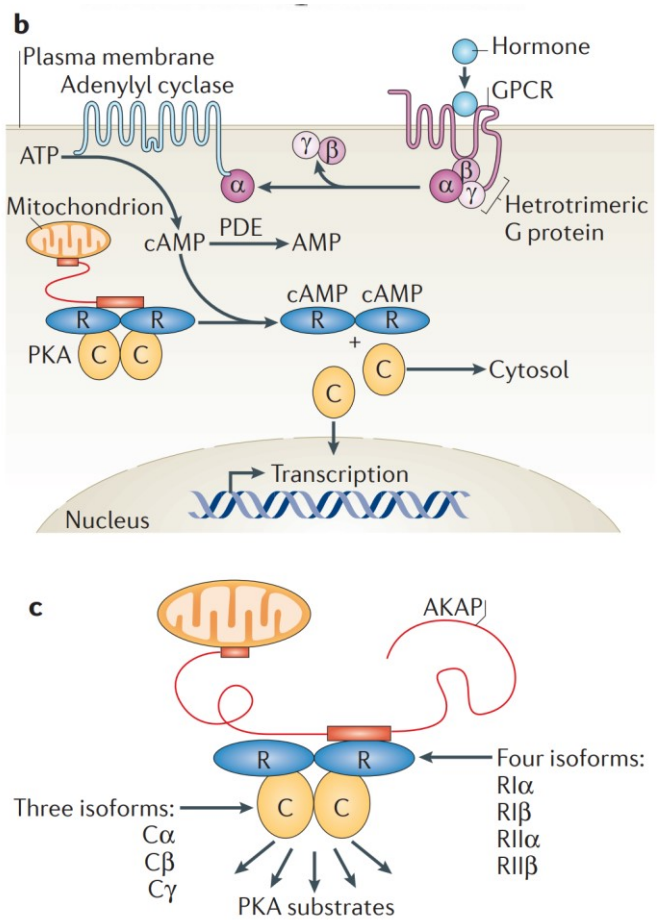

Figure L.3. Subunit conformation and intracellular working principle of physiological PKA.

The PKA signalling system includes the regulatory (R) and catalytic (C) subunits, PKA substrates and targeting proteins (such as A kinase anchoring proteins (AKAPs)) that that localize PKA to specific sites in the cell in close proximity to dedicated substrates

(Adopted by Taylor SS et al, 2012, Nature Reviews Molecular Cell Biology, Box 1)

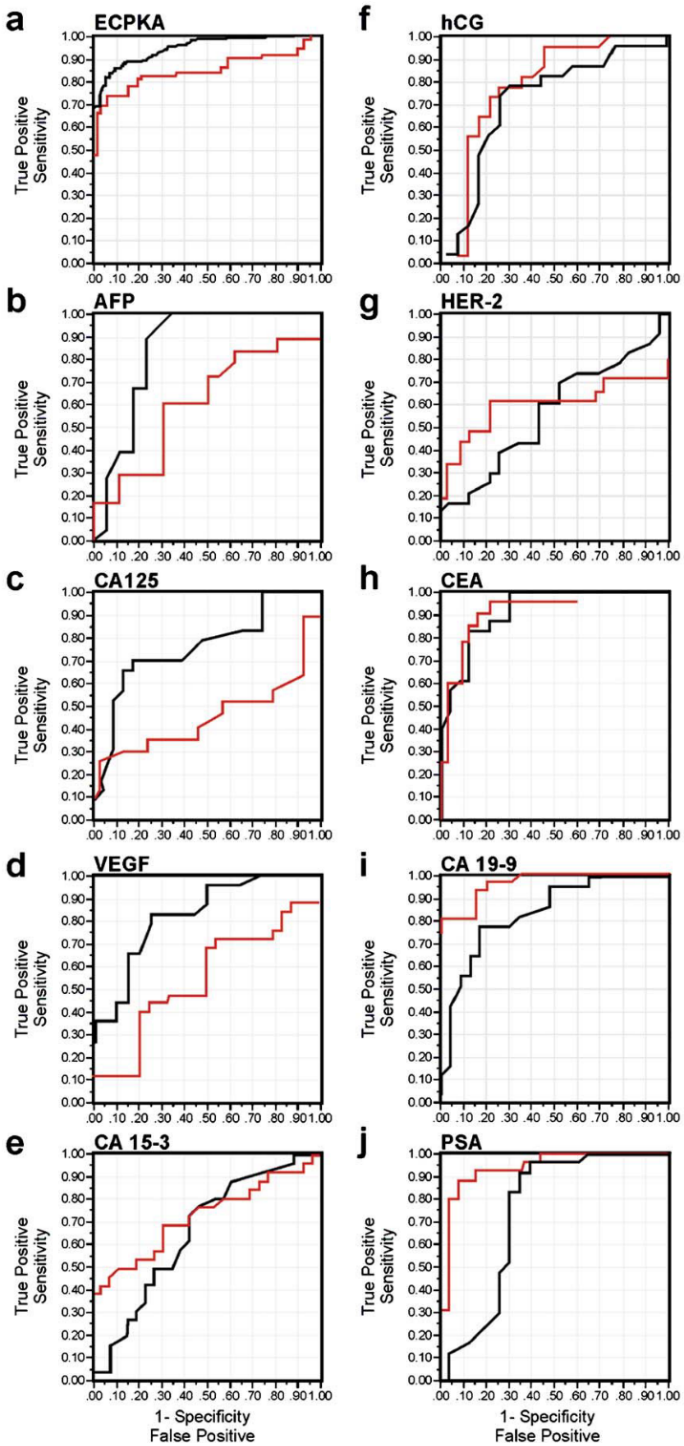
a
ECPKA
b
AFP
c
CA125
d
VEGF
e
CA 15-3
f
hCG
g
HER-2
h
CEA
i
CA 19-9
j
PSA
True Positive
Sensitivity
1- Specificity
False Positive

Figure L.4. Receiver operating characteristic plots of cancer biomarker and their autoantibody.

EIA and antigen EIA kit for serum tumor markers. The sensitivity and specificity of the autoantibody EIA and antigen EIA kit for each tumor marker are presented graphically in an ROC curve. Panel a represents the data of the ECPKA enzymatic assay (red) and those of the anti-ECPKA autoantibody EIA (black). In panels b-j: red=data from antigen-EIA kits; black=data of autoantibody EIA for each designated serum tumor markers. B, AFP; c, CA125; d, VEGF; e, CA15-3; f, hCG; g, HER-2; h, CEA; I, CA19-9; and j, PSA.

AFP, alpha-fetoprotein; CA, cancer antigen; CEA, carcinoembryonic antigen; ECPKA, extracellular protein kinase A; EIA, enzyme-immunoassay; hCG, human chorionic gonadotropin; HER, human epidermal growth factor receptor; PSA, prostate specific antigen; ROC, receiver operating characteristic; VEGF, vascular endothelial growth factor

(Adopted by Fig 3 of Nestrova M et al, 2006, BBA)

CHAPTER I

Extracellular cyclic AMP-dependent protein kinase A autoantibody and C-reactive protein as serum biomarkers for diagnosis of cancer in dogs

Abstract

Protein kinase A, a cyclic AMP-dependent enzyme, normally exists within mammalian cells; however, in cancer cells, it can leak out and be found in the serum. Extracellular cyclic AMP-dependent protein kinase A (ECPKA) has been determined to increase in the serum of cancer-bearing dogs. However, there have been no reports in the veterinary literature on serum ECPKA autoantibody (ECPKA-Ab) expression in dogs with cancer. The aim of this study was to evaluate ECPKA-Ab and C-reactive protein (CRP) as serum biomarkers for cancer in dogs. ECPKA-Ab and CRP levels were detected by an enzyme-linked immunosorbent assay in serum samples from dogs with malignant tumors (n = 167), benign tumors (n = 42), or non-tumor disease (n = 155) and from healthy control dogs (n = 123). ECPKA-Ab and CRP levels were significantly higher in the dogs with malignant

tumors than in those with benign tumors or non-tumor diseases, as well as in the healthy controls ($p<0.001$, Kruskal-Wallis test). There was a significant positive correlation between the neoplastic index, which was developed using ECPKA-Ab and CRP levels, and the presence of cancer in dogs ($p<0.001$); the area under the receiver operating characteristic curve was estimated to be >0.85 ($p<0.001$). In conclusion, ECPKA-Ab is a potential serum biomarker for a broad spectrum of cancers. Combined measurement of CRP and ECPKA-Ab levels in serum improves the sensitivity and accuracy of a diagnosis of cancer in dogs.

Keywords**:** autoantibody, biomarker, cancer, C-reactive protein, extracellular protein kinase A

1.1. Introduction

An early cancer diagnosis is important not only in humans (Allemani et al., 2015, Siegel et al., 2016) but also in companion animals (MacEwen, 1990), and can lead to better quality of life and longevity by allowing early and appropriate treatment (Allemani et al., 2015, Siegel et al., 2016). However, most cancers in dogs are detected by imaging or histopathology when it is too late to treat them.

Protein kinase A (PKA) is a cyclic AMP-dependent enzyme that participates in the proliferation, differentiation, metabolism, and apoptosis of cells in mammals (Kotani, 2012). PKA can leak out of cancer cells; when this occurs, it is known as extracellular cyclic AMP-dependent protein kinase A (ECPKA) (Cho et al., 2000b). ECPKA has been found to be higher in serum samples from human patients with cancer than in those from individuals without cancer, suggesting its potential as a powerful diagnostic marker of cancer in humans (Cho et al., 2000a, Cho et al., 2000b, Kita et al., 2004, Wang et al., 2007). In a previous study, the authors demonstrated a significant elevation in ECPKA levels in serum samples from dogs with cancer and suggested that ECPKA could be an important candidate diagnostic biomarker of canine malignancy (Bhang et al., 2017). However, although ECPKA is a powerful predictor of canine cancer, it is a fragile enzyme in certain situations. For example, it has only 20% activity after two freeze-thaw cycles (Cho

et al., 2000a). Therefore, detection of ECPKA autoantibodies (ECPKA-Ab) in serum has been investigated for a number of years in human medicine, and it has been demonstrated that an elevated serum ECPKA-Ab level has better diagnostic value for cancer than ECPKA alone (Nesterova et al., 2006b). However, as of yet, there have been no reports in the veterinary literature on serum ECPKA-Ab expression in dogs with cancer. Therefore, I have been interested in whether the serum ECPKA-Ab level could be a stable and accurate diagnostic biomarker of cancer in dogs.

C-reactive protein (CRP) is well known to be secreted in the acute phase of inflammation and is also a marker of cancer (Weinstein et al., 1984, Toniatti et al., 1990). CRP levels have been shown to be significantly higher in human patients with colorectal, lung, prostate, and breast cancers than in controls (Erlinger et al., 2004, Pine et al., 2011, Mahmoud and Rivera, 2002, Siemes et al., 2006). Various hypotheses have been put forward to explain why inflammation is a risk factor for cancer and why it occurs when cancer progresses (Siemes et al., 2006). Several veterinary oncology researchers have suggested that elevation of serum CRP in not necessarily diagnostic of cancer in dogs (Mischke et al., 2007, Nakamura et al., 2008), even though CRP expression has been found to be higher in dogs with malignancy than in those without the disease and to indicate the stage of cancer progression (Planellas et al., 2009, Nielsen et al., 2007). However, Selting et al.

demonstrated that the serum thymidine kinase 1 activity and CRP levels in the serum of dogs with cancer have high diagnostic value as markers of canine malignancy (Selting et al., 2015a, Selting et al., 2016b, Thamm et al., 2012). They showed that the thymidine kinase 1 activity was high in dogs with hemangiosarcoma and other malignant canine tumors and that a combination of thymidine kinase 1 activity and CRP expression levels had increased the diagnostic accuracy for detection of cancer. Therefore, CRP might be used as an adjunct to other serum biomarkers to improve our ability to diagnose cancer in dogs.

The objectives of this study were to evaluate the diagnostic significance of ECPKA-Ab in canine malignancy and to determine if combined measurement of CRP and ECPKA-Ab levels in serum improves diagnostic accuracy for detection of cancer in dogs.

1.2. Materials and Methods

Animals and samples

Four hundred and eighty-seven privately owned dogs (123 control dogs with no known disease, 42 with benign tumors, 167 with malignant tumors, and 155 with non-tumor disease) were included in the study. Serum samples were provided by Seoul National University Veterinary Medical Teaching Hospital and several local animal clinics in the Republic of Korea. All of the dogs included in this study underwent a physical examination; blood examination including complete blood counts, serum biochemical profiles, and electrolytes; and diagnostic imaging such as abdominal sonography, radiography (thoracic and abdominal), and an optional echocardiogram or computed tomography. Malignant tumors were diagnosed by cytology or/and histopathology. One hundred and twenty-three controls with no known disease visited the animal clinic for neutralization or regular checkup and were confirmed to have no abnormalities based on medical examination. All experiments were approved by and followed the policies and regulations of the Laboratory Animals Institutional Animal Care and Use Committee (SNU-180130-2; Seoul National University, Seoul, Korea).

ECPKA autoantibody ELISA

The presence and level of ECPKA-Ab in canine serum samples were

assessed by enzyme-linked immunosorbent assay (ELISA). First, 100 µL of canine serum samples diluted 500-fold in reagent diluent were added to 96-well ELISA plates precoated with canine PKA Cα and incubated for 1 hour at room temperature. The plates were then incubated further with anti-dog immunoglobulin G:horseradish peroxidase antibodies for 1 hour at room temperature in a dark room. Next, the plates were developed with 3,3',5,5'-tetramethylbenzidine substrate solution for 15 min at room temperature. The reaction was stopped with 50 µL of stop solution. Absorbance was determined at 450 nm using a scanning multi-well spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Canine CRP ELISA

The serum CRP concentration was assessed using a canine CRP ELISA kit (Abcam, Cambridge, UK) following the manufacturer's instructions. Briefly, 100 µL of canine serum samples diluted in reagent diluent were added to 96-well ELISA plates precoated with anti-canine CRP antibodies and incubated for 10 minutes at room temperature. After washing, 100 µL of the enzyme-antibody conjugate were added to the plates, followed by incubation for 10 minutes at room temperature in the dark. The plates were then developed with 3,3',5,5'-tetramethylbenzidine substrate solution for 5 minutes at room temperature, and the reaction was stopped with 100 µL of stop solution. Absorbance was determined at 450 nm with a

scanning multi-well spectrophotometer (Molecular Devices).

Statistical analysis

The data were tested for normality using the Shapiro-Wilk test. Differences between more than two groups were analyzed using one-way ANOVA, and the differences between two groups were analyzed using the t-test, all of which were in parametric distribution. The Bonferroni test was used as post-hoc analysis after the one-way ANOVA test. Differences between more than two groups were analyzed using the Kruskal-Wallis test and differences between two groups were analyzed using the Mann-Whitney test—all in non-parametric distribution. Dunn's multiple comparisons test was used as post-hoc analysis after Kruskal-Wallis test. A neoplastic index (NI) was developed by binary logistic regression based on the ECPKA-Ab and CRP levels. The statistical program, SPSS for Windows version 23 (IBM Corp., Armonk, NY, USA), was used to create a multivariable equation using ECPKA-Ab and CRP as the individual variables and the neoplastic index as the result. The cut-off value for discrimination between the two groups was estimated by analyzing the receiver-operating characteristic (ROC) curve. All graphs are presented as box and whisker plots. All the data are shown as the median and range obtained in at least three independent experiments. The statistical analyses were performed using SPSS and GraphPad Prism version 7 (GraphPad

Software, Inc, La Jolla, CA, USA). A p-value of <0.05 was considered statistically significant.

1.3. Results

I tested serum samples from 167 dogs with a diagnosis of cancer and 320 dogs without a diagnosis of cancer, including those that were considered to be healthy or with a benign tumor or non-tumor disease. The signalment data are summarized in Table 1.1. The median age of the total study population was 10.00 (range: 0.25–18.00) years and the main breeds were Maltese (n = 95) and Shih-tzu (n = 71). The cancers were categorized according to the cell of origin—carcinoma, sarcoma, hematopoietic lymphoreticular, or neuroendocrine. The final diagnoses in the group of dogs with cancer (n = 167) are shown in Table 1.2. The cancer group included 98 dogs with carcinoma (malignant mammary gland tumor [MMGT], transitional cell carcinoma [TCC], hepatocellular carcinoma [HCC], pulmonary adenocarcinoma [PAC]), 33 with sarcoma (melanoma [Mel], hemangiosarcoma [HSA], soft tissue sarcoma), 35 with hematopoietic/lymphoreticular disease (lymphoma and leukemia), and one with a neuroendocrine tumor (pheochromocytoma). The 3 dogs with leukemia had either chronic lymphocytic leukemia (n = 2) or acute megakaryocytic leukemia (n = 1). The diseases in the 155 dogs in the non-tumor group are categorized according to organ system in Table 1.3. The most common diseases were cardiovascular (n = 42) and urologic (n = 30).

The serum ECPKA-Ab level was analyzed by ELISA (Figure 1.1). The

median ECPKA-Ab levels in the cancer, benign tumor, non-tumor disease, and healthy control groups were 6540.0 (range: 1665–31,900) ng/mM, 3995.0 (range: 1875–8870) ng/mL, 3695.0 (range: 890–11,900) ng/mL, and 3717.6 (range: 1455–7153) ng/mL, respectively. The median ECPKA-Ab level in the cancer group was significantly higher than the levels in the benign tumor, non-tumor disease, and healthy groups (all $p<0.0001$, Kruskal-Wallis test). The median ECPKA-Ab levels in the dogs with carcinoma, sarcoma, and hematopoietic/lymphoreticular disease were 6910 (range 2040–31,900) ng/mL, 6165 (range 2720–30,460) ng/mL, and 6690 (range 1665–19,820), respectively, and the level in the dog with the neuroendocrine tumor was 6440 ng/mL. Higher median ECPKA-Ab levels were detected in the dogs with diagnoses of MMGT (7270 ng/mL), lymphoma (6418 ng/mL), HCC (8275) ng/mL, TCC (6310 ng/mL), HSA (5820 ng/mL), PAC (6258 ng/mL), and Mel (6100 ng/mL).

Dog breed did not appear to have a significant effect on the ECPKA-Ab level in dogs with or without cancer ($p = 0.621$, and $p = 0.204$, respectively). There was no significant difference between male and female dogs in both the cancer and non-cancer groups ($p = 0.557$, and $p = 0.624$, respectively). To analyze the effect of neutralization, ECPKA-Ab levels were compared in both castrated and non-castrated male dogs and in both spayed and non-spayed female dogs, respectively. In the dogs with cancer, there was no significant difference between spayed and

non-spayed female dogs in terms of ECPKA-Ab levels ($p = 0.08$); the number of male dogs in the cancer group was too small to analyze the effect of castration. In the non-cancer dogs, there was no difference between spayed and non-spayed female dogs ($p = 0.128$) or between the castrated and non-castrated male dogs ($p = 0.778$) in terms of ECPKA-Ab levels.

The CRP levels in the canine serum samples were analyzed by ELISA (Figure 1.1). The median CRP levels in the cancer, benign tumor, non-tumor disease, and healthy groups were 12.64 (range: 0.5–348) mg/L, 4.61 (range: 1.1–178.8) mg/L, 3.47 (range: 0–170.4) mg/L, and 2.3 (range: 0.27–18.03) mg/L, respectively. The CRP level in the cancer group was significantly higher than the levels in the benign tumor, non-tumor disease, and healthy groups (all $p<0.0001$, Kruskal-Wallis test). The median CRP levels in the carcinoma, sarcoma, and hematopoietic/lymphoreticular disease subgroups were 11.57 (range: 0.52–313.3) mg/L, 27.56 (range: 1–348) mg/L, and 11.9 (range: 0.5–243) mg/L, respectively; the CRP level in the dog with the neuroendocrine tumor was 0.9 mg/L. Higher CRP levels were detected in the dogs with diagnoses of MMGT (18.45 mg/L), lymphoma (13.24 mg/L), HCC (26.85 mg/L), TCC (3.68 mg/L), HSA (15.91 mg/L), PAC (8.69 mg/L), and Mel (28 mg/L).

The NI derived from the ECPKA-Ab and CRP levels is shown in Figure 1.1E and 1.1F. The NI was higher in the cancer group than in the benign tumor,

non-tumor disease, and healthy study groups (all $p<0.001$, Kruskal-Wallis test). The subcategories in the cancer group had significantly high NI values (Figure 1.1F).

A ROC analysis was then performed to evaluate the value of ECPKA-Ab and NI as diagnostic biomarkers of cancer when all dogs in the study population were classified according to whether they did or did not have cancer (Figure 1.2). The non-cancer group included the benign tumor, non-tumor disease, and healthy study groups. Both the ECPKA-Ab level and the NI value were significantly higher in the cancer group (both $p<0.001$, Mann-Whitney U-test). The area under the receiver-operating characteristic curve (AUROC) was 0.86 for the ECPKA-Ab level and 0.89 for the NI value. The diagnostic accuracy of the combination of ECPKA-Ab and NI is shown in Table 1.4.

To determine whether the ECPKA-Ab level or NI increases in certain diseases, the values for these parameters were analyzed by type of disease (Figure 1.3); there was no significant difference in either of these values between any of the disease groups and the healthy group (Kruskal-Wallis test with Dunn's multiple comparisons test, $p = 0.1395$).

1.4. Discussion

The purpose of this study was to determine the presence and level of ECPKA-Ab in canine serum and to evaluate the value of ECPKA-Ab as a biomarker of canine cancer. ECPKA-Ab were detected in canine serum and at higher levels in the cancer group than in the non-cancer group. The CRP level was also increased in the cancer group, and the NI, developed by regression analysis of both ECPKA-Ab and CRP, had a higher AUROC than ECPKA-Ab alone when both NI and ECPKA-Ab had an AUROC >0.85.

The finding of a significantly higher serum ECPKA-Ab level in dogs with cancer in the present study (Figures 1.1 and 1.2) is consistent with a previous finding in dogs (Bhang et al., 2017). Moreover, when the cancers were classified, as shown in Table 1.2, the ECPKA-Ab level was found to be increased regardless of the cell of origin (Figure 1.1B) and was significantly higher in the cancer group than in the healthy group; this finding has also been reported in human patients (Nesterova et al., 2006a). There are a number of other studies in the human literature that report the ECPKA-Ab level being increased in patients with various types of cancer (Choi et al., 2006, Loilome et al., 2012, Zaenker and Ziman, 2013). Therefore, the ECPKA autoantibody is regarded as a universal cancer biomarker (Zaenker and Ziman, 2013). Whereas other specific biomarkers can detect only one

specific type of cancer, e.g., prostate-specific antigen for prostate cancer and α-fetoprotein for liver cancer (Zaenker and Ziman, 2013), ECPKA-Ab can be used to detect various types of malignancies. Furthermore, based on my findings, it may be possible for ECPKA-Ab to be used as a screening marker for occult cancer, considering that cancer in most dogs progresses subclinically and frequently goes undetected using conventional blood tests. It is ideal for the screening biomarker used to diagnose occulting cancer to have a high sensitivity >90%, but considering that most dogs with cancer are diagnosed at the late stages—when clinical symptoms have begun to manifest—this marker could be a clinically meaningful biomarker. Additionally, the ECPKA-Ab level can be used to help predict the risk of malignancy in dogs with masses already detected by physical examination or imaging, as this level is not increased in the serum of dogs with benign tumors (Figure 1.1). Biopsy is the standard procedure for confirming whether a mass is cancerous or benign, however, it has several limitations in clinical use, including issues with anesthesia, hemorrhage in coagulopathy, and accessibility. Cytology is a simple diagnostic method that is used clinically, and though it has a high specificity, the sensitivity is low. Therefore, ECPKA-Ab levels can provide additional information to determine whether the mass is malignant or benign.

In this study, I did not compare the ECPKA and ECPKA-Ab levels within the same samples, so it was not possible to compare the diagnostic accuracy of

ECPKA with that of ECPKA-Ab. However, given the findings of studies in humans (Nesterova et al., 2006b, Nesterova et al., 2006a), the diagnostic accuracy of ECPKA-Ab would be expected to be better than that of ECPKA in dogs. The diagnostic accuracy achieved using autoantibodies of several biomarkers of cancer, e.g., α-fetoprotein (liver cancer) and CA125 (ovarian cancer), has been compared with that of the biomarkers alone in the detection of cancer (Nesterova et al., 2006a). The presence of ECPKA-Ab in human serum has been confirmed, and its usefulness in the diagnosis of human cancer has been suggested (Nesterova et al., 2006a, Choi et al., 2006, Loilome et al., 2012).

In this study, the NI had a higher AUROC and higher sensitivity, specificity, and accuracy than ECPKA-Ab alone (Figure 1.2, Table 1.4), indicating that ECPKA-Ab is a more powerful biomarker of cancer when combined with CRP. This result is very similar to the findings for thymidine kinase 1 activity reported by Selting et al (Selting et al., 2015a, Selting et al., 2016b). In their studies, thymidine kinase 1 activity was used as a serum biomarker of cancer in dogs, and its diagnostic accuracy was improved when used with CRP. As in other studies that have demonstrated an increased CRP level in dogs with cancer (Mischke et al., 2007, Nakamura et al., 2008), I found a significantly higher CRP level in dogs with cancer than in those without cancer (Figure 1.1C, 1.1D). However, because CRP shows a nonspecific increase in various situations, including inflammation (Nakamura et al.,

2008), it could not be used as a sole biomarker for cancer. Although elevated CRP is not in itself a biomarker of cancer, it may serve as a co-factor to increase the diagnostic accuracy of other biomarkers in detecting cancer.

The AUROC of both ECPKA-Ab and NI were 0.86 and 0.89, respectively. At the best cut-off point, which is determined as the point where the sum of sensitivity and specificity is highest (at the same time, when sensitivity is greater than specificity), positive predictive value (PPV) was rather low in both ECPKA-Ab and NI (Table 1.4). Although positive and negative predictive values (NPV) are changeable by included data, these markers have a relatively low PPV and a high NPV. This result is based on setting the cut-off point to where the sensitivity is high. These predictive values suggest that ECPKA-Ab and NI could be used as a rule-in biomarker, but not a rule-out biomarker. Similarly, bladder tumor antigen (BTA), a urine biomarker for transitional cell carcinoma (TCC), has a higher sensitivity (85-90%) and a much lower specificity (35-94.4%) (Billet et al., 2002, Henry et al., 2003); therefore, it can rule-in TCC, but not rule-out TCC. The low specificity of BTA results in the inability to distinguish bladder cancer from bacterial cystitis or hemorrhagic cystitis (Billet et al., 2002, Henry et al., 2003). On the other hand, the TK1 activity test and B-raf proto-oncogene serine/threonine kinase gene mutation test have high specificities and low sensitivities, suggesting that it can be used to confirm malignant tumors (Selting et al., 2015a, Selting et al., 2016b, Thamm et al.,

2012, Mochizuki et al., 2015). A high sensitivity test is suitable as a screening tool, and depending on the result, additional tests may be needed to confirm the diagnosis. High-specificity tests can confirm cancer, but they cannot diagnose cancer if the result is negative.

To determine whether a specific variable other than cancer could lead to a high ECPKA-Ab level in canine serum, I analyzed the effect of sex, castration stratus, breed, and type of non-tumor disease. I found no significant effect of sex, castration status, or breed in this study. However, only a few breeds and a small number dogs per breed were included in this investigation; thus, it is possible that another result could be obtained in a different study sample with different breeds. Furthermore, there was no significant variation in the ECPKA-Ab level according to type of disease in the non-tumor disease group (Figure 1.3). However, the spectrum of disease included in this patient group was not necessarily representative, so the ECPKA-Ab level should be further investigated in various diseases. In particular, immune-mediated diseases that result from self-perpetuating B-cells and autoantibodies (Edwards et al., 1999) need to be studied in more detail to assess their potential effect on the ECPKA-Ab level. Moreover, the main immune-mediated disease in this study was atopic dermatitis with lesser numbers of patients with immune-mediated hemolytic anemia or thrombocytopenia. Studies that include larger numbers of patients with these and other autoimmune diseases are

needed.

The causes of false-negative canine cases, i.e., dogs with cancer and low ECPKA-Ab levels, also need to be investigated. ECPKA-Ab consist mainly of immunoglobulin G, which has antibody-forming ability in the body and could affect the ECPKA-Ab level. It is presumed that if a dog with cancer is immunosuppressed, its ability to form antibodies would decrease and the ECPKA-Ab level would be low. Furthermore, follow-up investigations are required to confirm the relationship between occult cancer and survival. Additional studies are also needed to determine whether the ECPKA-Ab level and the NI value can be used for therapeutic monitoring.

In summary, I have shown that the ECPKA-Ab level and the NI value are meaningful serum biomarkers of canine cancer. In this study, dogs with cancer had higher ECPKA-Ab levels than those without cancer and a combination of the ECPKA-Ab and CRP levels had increased the diagnostic accuracy for detecting cancer. Therefore, this combination of biomarkers may help to improve the efficacy of cancer treatment by increasing the diagnosis rate—even in dogs with cancer that appear clinically healthy.

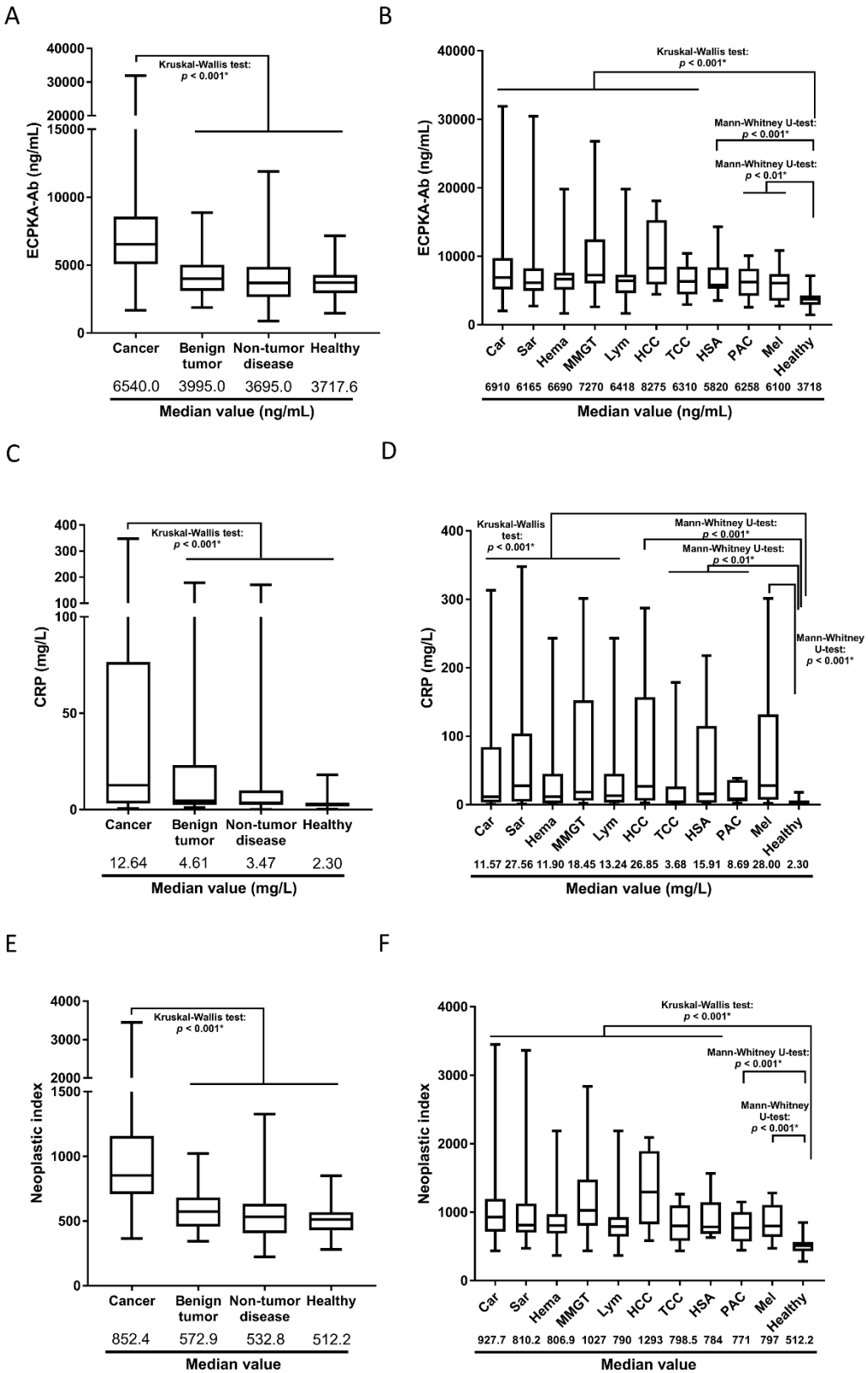
A
ECPKA-Ab (ng/mL)
Kruskal-Wallis test: p < 0.001*
Cancer
Benign tumor
Non-tumor disease
Healthy
6540.0
3995.0
3695.0
3717.6
Median value (ng/mL)
B
ECPKA-Ab (ng/mL)
Kruskal-Wallis test: p < 0.001*
Mann-Whitney U-test: p < 0.001*
Mann-Whitney U-test: p < 0.01*
Car
Sar
Hema
MMGT
Lym
HCC
TCC
HSA
PAC
Mel
Healthy
6910 6165 6690 7270 6418 8275 6310 5820 6258 6100 3718
Median value (ng/mL)
C
CRP (mg/L)
Kruskal-Wallis test: p < 0.001*
Cancer
Benign tumor
Non-tumor disease
Healthy
12.64
4.61
3.47
2.30
Median value (mg/L)
D
CRP (mg/L)
Kruskal-Wallis test: p < 0.001*
Mann-Whitney U-test: p < 0.001*
Mann-Whitney U-test: p < 0.01*
Mann-Whitney U-test: p < 0.001*
Car
Sar
Hema
MMGT
Lym
HCC
TCC
HSA
PAC
Mel
Healthy
11.57 27.56 11.90 18.45 13.24 26.85 3.68 15.91 8.69 28.00 2.30
Median value (mg/L)
E
Neoplastic index
Kruskal-Wallis test: p < 0.001*
Cancer
Benign tumor
Non-tumor disease
Healthy
852.4
572.9
532.8
512.2
Median value
F
Neoplastic index
Kruskal-Wallis test: p < 0.001*
Mann-Whitney U-test: p < 0.001*
Mann-Whitney U-test: p < 0.001*
Car
Sar
Hema
MMGT
Lym
HCC
TCC
HSA
PAC
Mel
Healthy
927.7 810.2 806.9 1027 790 1293 798.5 784 771 797 512.2
Median value

Figure 1.1. Enzyme-linked immunosorbent assay data for the ECPKA autoantibody, CRP, and NI values in each study group. (A) ECPKA-Ab levels in the cancer, benign tumor, non-tumor disease, and healthy study groups. (B) ECPKA-Ab levels in the different cancer subgroups, including carcinoma, sarcoma, hematopoietic/lymphoreticular disease, and the subcategories therein. (C) CRP levels in the cancer, benign tumor, non-tumor disease, and healthy study groups. (D) CRP levels in the different cancer subgroups, including carcinoma, sarcoma, hematopoietic/lymphoreticular disease, and the subcategories therein. (E) The NI value in the cancer, benign tumor, non-tumor disease, and healthy study groups. (F) The NI value in the different cancer subgroups, including carcinoma, sarcoma, hematopoietic/lymphoreticular disease, and the subcategories therein. All graphs are shown as box and whisker plots. Each box includes the interquartile range; the line within each box represents the median and the whiskers represent the range, extending to a maximum of 1.5 times the interquartile range. Abbreviations: Ab, antibodies; Car, carcinoma; CRP, C-reactive protein; ECPKA, extracellular cyclic AMP-dependent protein kinase; HCC, hepatocellular carcinoma; Hema, hematopoietic/lymphoreticular; HSA, hemangiosarcoma; Lym, lymphoma; Mel, malignant melanoma; MMGT, malignant mammary gland tumor; Mel, malignant melanoma; NI, neoplastic index; Sar, sarcoma; TCC, transitional cell carcinoma; Mel, malignant melanoma

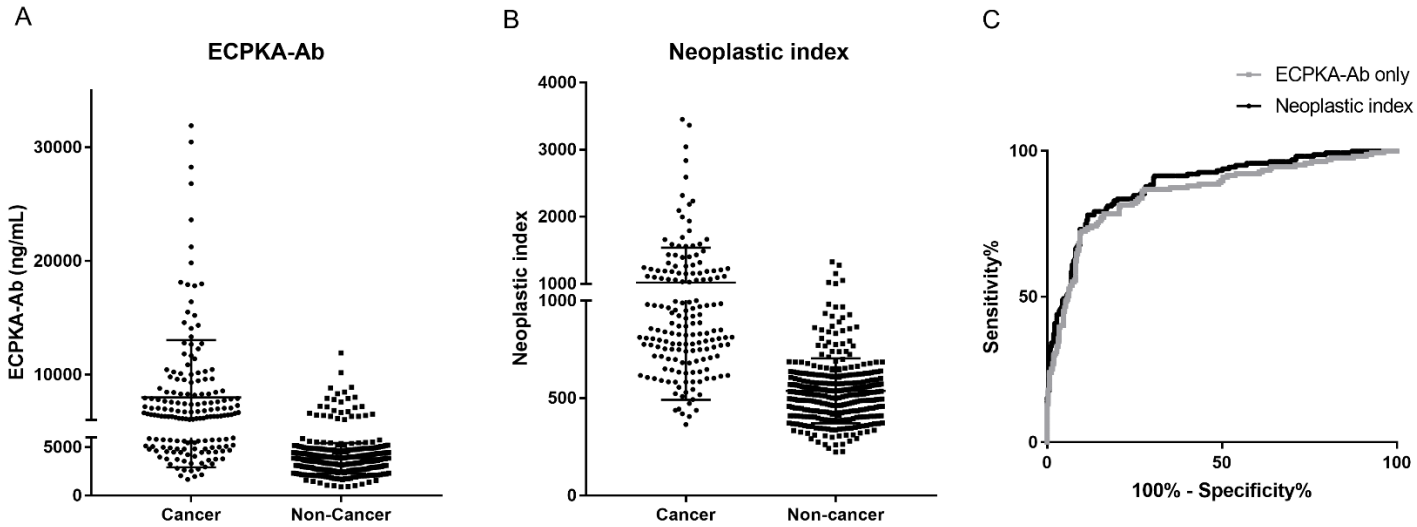

Figure 1.2. Differences in the ECPKA-Ab level and NI value between dogs with and without a diagnosis of cancer. (A) ECPKA-Ab levels. (B) NI levels. (C) Receiver-operating characteristic curve for ECPKA-Ab and NI. All error bars represented in this figure are shown as median with interquartile range. Abbreviations: Ab, antibodies; ECPKA, extracellular cyclic AMP-dependent protein kinase; NI, neoplastic index

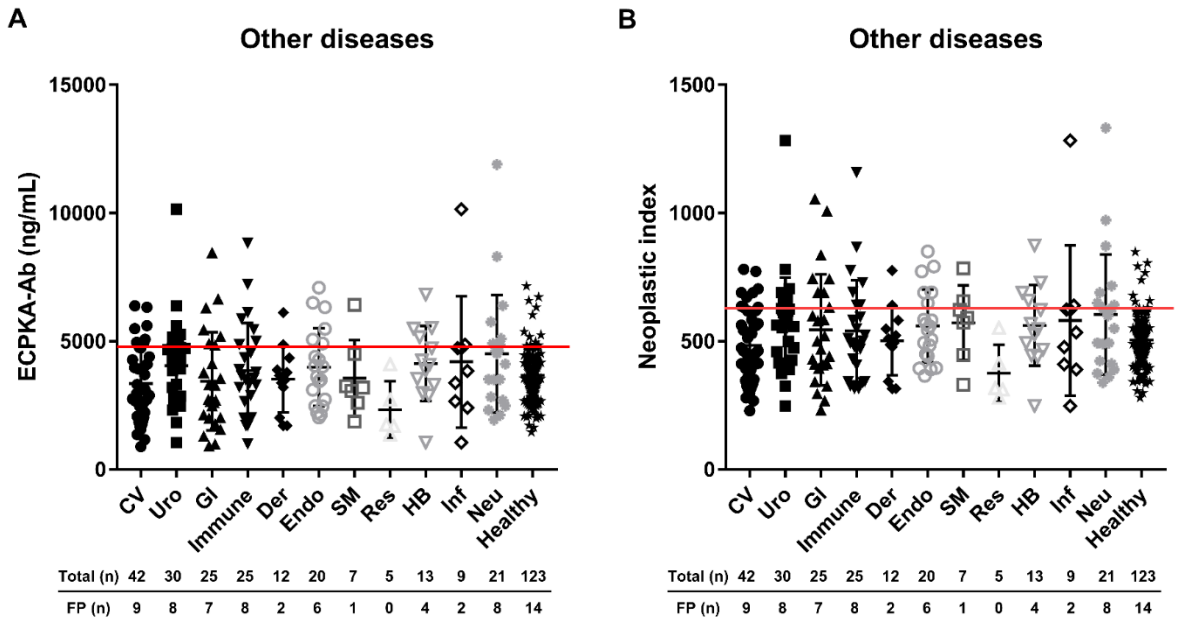

Figure 1.3. Distribution of ECPKA-Ab and NI when the diseases were classified according to organ system. A scatter plot of other disease group and healthy group was shown in case of (A) ECPKA-Ab levels and (B) NI value. The data are shown as the mean and standard deviation. The number of dogs are shown as footnotes. Abbreviations: Ab, antibodies; CV, cardiovascular; Der, dermatologic; ECPKA, extracellular cyclic AMP-dependent protein kinase; Endo, endocrine; FP, false positive; GI, gastrointestinal; HB, hepatobiliary; Immune, immune-mediated; Inf, infectious; SM, skeletomuscular; Neu, neurologic; NI, neoplastic index; Res, respiratory; Uro, urologic

Table 1.1. Signalment data for dogs included in this study.

	Normal	Non-tumor diseases	Benign tumor	Malignant tumor
N	123	155	42	167
Median age (range)	3.00 (0.25–16.00)	10.92 (0.25–18.00)	10.46 (0.50–16.70)	13.00 (0.59–18.00)
Sex (N)	CM (25), F (47), M (29), SF (22)	CM (67), F (18), M (9), SF (61)	CM (18), F (11), M (1), SF (12)	CM (64), F (24), M (3), SF (76)
Breed (N)	Beagle (60), Miniature Poodle (15), Maltese (10), Pomeranian (7), Bichon Frise (5), Other (26)	Maltese (46), Shih-tzu (19), Miniature Poodle (17), Mongrel (12), Miniature Schnauzer (11), Cocker Spaniel (10), Yorkshire Terrier (10), Other (30)	Cocker Spaniel (10), Maltese (8), Shih-tzu (4), Miniature Poodle (4), Other (16)	Shih-tzu (47), Maltese (29), Cocker Spaniel (25), Mongrel (12), Yorkshire Terrier (11), Other (43)

CM, castrated male; F, female; M, male; SF, spayed female

Table 1.2. Types of cancer and numbers of affected dogs.

Type of cancer (cell origin)	Cancer (N)	Total (N)
Carcinoma (epithelial)	Malignant MGT (29), TCC (19), HCC(9), pulmonary adenocarcinoma (6), RCC (4), adenocarcinoma of the prostate (4), ASAC (3), SCC (3), Other (21)	98
Sarcoma (mesenchymal)	Hemangiosarcoma (9), melanoma (9), soft tissue sarcoma (4), other (10)	33
Hematopoietic or lymphoreticular	Lymphoma (28), leukemia (3), other (4)	35
Neuroendocrine	Pheochromocytoma (1)	1

MGT, mammary gland tumor; TCC, transitional cell carcinoma; HCC, hepatic cellular carcinoma; RCC, renal cell carcinoma; ASAC, anal sac adenocarcinoma; SCC, squamous cell carcinoma

Table 1.3. Disease types and numbers of dogs with non-tumor diseases in this study.

Types of non-tumor disease	N
Cardiovascular	42
Urologic	30
Gastrointestinal	25
Immune-mediated	25
Neurologic	21
Endocrine	20
Hepatobiliary	13
Dermatologic	12
Infectious	9
Musculoskeletal	7
Respiratory	5

Table 1.4. Diagnostic ability of ECPKA-Ab and neoplastic index.

	ECPKA-Ab	NI
AUROC	0.86	0.89
95% Cl	0.82–0.90	0.85–0.92
P-value	<0.0001	<0.0001
Sensitivity	80.84	82.93
Specificity	79.38	80.88
Accuracy	79.88	81.57
PPV	67.16	69.04
NPV	88.81	90.21

AUROC, area under the receiver-operating characteristic curve; ECPKA-Ab, extracellular cyclic AMP-dependent protein kinase autoantibodies; NI, neoplastic index; NPV, negative predictive value; PPV, positive predictive value

Chapter II

Changes in concentrations of extracellular cyclic AMP-dependent protein kinase A and its autoantibody depending on tumor size *in vitro* and *in vivo* mouse model

Abstract

High level of ECPKA and ECPKA-Ab are present in serum of cancer patients. The objective of this study was to examine whether ECPKA and its autoantibody levels are correlate to tumor size. *In vitro* study, Lewis lung carcinoma (LLC1) cell line was seeded at $4x10^5$ cells and $8x10^5$ cells respectively in 6-well culture plate. After 24hr, cells were retracted and mRNA level of PRKARIA, PRKARIIB and PRKACA was analyzed by qRT-PCR. PKA level in cellular protein and conditioned medium was analyzed by western blot and ELISA. For developing cancer model, C57BL/6J mice were injected LLC1 $1x10^6$ cells diluted in 100ul of PBS subcutaneously. When tumor size reached at $100mm^3$, 300mm3, $600mm^3$, and $1,000mm^3$, blood and tumor tissue was collected from mice (n=6/group). As a result, all mRNA level of PRKARIA, PRKARIIB, and PRKACA was not different as

different cell number *in vitro* study. Both intracellular and extracellular PKA protein level were increased with increased cell numbers. *In vivo* study revealed that ECPKA-Ab levels were dependent on tumor size. Serum ECPKA-Ab levels were more accurate than ECPKA and it is dependent on tumor size.

Keywords: autoantibody, cisplatin, chemotherapy, protein kinase A, tumor size

2.1. Introduction

Cancer cells were determined to leak catalytic subunit of protein kinase A out of cells whereas physiologic protein kinase A exists in subcellular space (Cho et al., 2000b, Cho et al., 2000a). This leaked protein kinase A is called extracellular protein kinase A (ECPKA) (Cho et al., 2000b, Cho et al., 2000a). In human medicine, ECPKA was determined in serum of cancer patient and many researches showed ECPKA is present in serum of cancer patient in higher level comparing to healthy people (Cho et al., 2000b, Kita et al., 2004, Cvijic et al., 2000). ECPKA-Ab level also exist in high level in cancer patient (Cho-Chung, 2006, Nesterova et al., 2006b, Loilome et al., 2012) and ECPKA-Ab level is more sensitive and specific than ECPKA when comparing cancer patient to healthy people using receiver operating characteristic curves (Nesterova et al., 2006b). In the previous study, presence of ECPKA and ECPKA-Ab were determined in canine serum and dogs with cancer had higher level of ECPKA and ECPKA-Ab in their serum than healthy, disease suffering, and benign tumor bearing dogs (Bhang et al., 2017), (Chapter 1).

Cancer biomarker is defined as biological molecule produced by the tumor cell or biological molecule produced other tissues in response to cancer (Füzéry et al., 2013). Clinically, cancer biomarkers give one or several information about diagnosis, prognosis, screening, risk assessment, prediction of benefit from a specific therapy, pharmacodynamics (monitoring), and selection of anticancer

drugs (Henry and Hayes, 2012, Febbo et al., 2011). In the previous study, serum ECPKA and ECPKA-Ab was suggested as cancer biomarker for diagnosis and prognosis in dogs (Bhang et al., 2017), (Chapter 1). In human medicine, there a study showed that surgical removal of melanoma decreases serum ECPKA level (Kita et al., 2004) and another study showed decreased serum ECPKA-Ab level after chemotherapy in lymphoma patients (Choi et al., 2006). Considering the results of that papers, the possibility that ECPKA and ECPKA-Ab may be used as monitoring markers can be considered.

The objective of this study is to confirm relationship of both ECPKA and ECPKA-Ab versus cancer size and to evaluate possibility of ECPKA and ECPKA-Ab as monitoring markers. I hypothesize that increased cancer size induce higher level of ECPKA and ECPKA-Ab in serum. With Lewis lung carcinoma (LLC1) cell line, mRNA and intracellular/extracellular protein level of protein kinase A were determined at different seeding cell number *in vitro* study. Animal study with C57BL/6J mice designed to evaluate serum ECPKA and its autoantibody levels according to tumor size.

2.2 Materials and Methods

In vitro study

Lewis lung carcinoma (LLC1) cells (ATCC, Manassas, VA, USA) were cultured in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM; PAN Biotech, Aidenbach, Germany) with 10% fetal bovine serum (FBS; PAN Biotech, Aidenbach, Germany) in an incubator at 37℃ with 5% CO_2. Cells were seeded onto 6 well plates at a density of 4 x 10^5 cells/well, and 8 x10^5 cells/well, respectively. After 24hr, media was removed and changed to new 3 ml of media after washing with phosphate buffered saline (PBS; PAN Biotech, Aidenbach, Germany). After 48 hours, the conditioned media were aliquoted and stored in a freezer at -80℃ for later analysis and cells were detached, counted and processed to RNA extraction or protein extraction.

Animal Study

Total thirty male C57BL/6J-mice aged 7 weeks (Central Lab. Animal Inc., Seoul, Republic of Korea) were used for study relationship between tumor size and ECPKA/ECPKA-Ab levels. Summary of study design are showed in Figure 2.1. Mice were injected subcutaneously with 1x10^6 LLC1 cells at day 0. Tumors were measured daily using calipers and volume was calculated with equation "0.52 x length x width^2" (length > width). For study relationship between tumor size and ECPKA/ECPKA-Ab levels in serum, thirty male C57BL/6J-mice aged 7 weeks were randomized to five groups of 6 mice: control, 100 mm^3 tumor, 300 mm^3 tumor, 600 mm^3 tumor, 1000 mm^3 tumor. After injecting LLC1, respective groups were

sacrifice at tumor size 100 mm^3, 300 mm^3, 600 mm^3, and 1000 mm^3 after blood sampling. Control group was sacrificed after blood sampling at day 0.

Real-time polymerase chain reaction

mRNA level of genes related to protein kinase A including PRKARIA, PRKARIIB, and PRKACA in LLC1 cells was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted using QIAGEN RNeasy Mini kit (Qiagen, Hilden, Germany) and cDNA was synthesized from 1ug of total RNA using Cellscript All-in-One 5X first strand cDNA Synthesis Master Mix (CellSafe, Seoul, Korea). qPCR was performed using the StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA) with AMPIGENE qPCR Green Mix Hi-Rox (Enzo Life sciences, NY, USA). All primers were designed using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are summarized in Table 2.1.

Protein extraction

Proteins were extracted from LLC1 cells and mouse tumor tissues using PRO-PREP Protein Extraction Solution (iNtRON, Gyeong-gi, Korea) following the manufacturer's instruction. Briefly, cultured LLC1 cells were detached after 48 hours with 0.5% trypsin-EDTA and tumor tissues were obtained from tumor-bearing mice after sacrifice. Cell pellets were obtained by centrifugation of detached LLC1 cells and PRO-PREP solution was added into the cell pellets. 10mg of tumor tissues were obtained after weighing whole tumor tissues, and were homogenized by tissue homogenizer after adding PRO-PREP solution. The

mixtures of PRO-PREP solution with cells or tissues were incubated for 20 minutes on freezer at -20℃. After centrifugation, the supernatants were obtained.

Western blot

Proteins were extracted from LLC1 cells, resolved on SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The transferred membranes were blocked with 5% skim milk in Tris-buffered saline-Tween 20 (TBST: 0.1% Tween 20, 100 mM NaCl and 10 mM Tris-HCL, (pH 7.6)) for 1 h at room temperature. Blots were incubated with antibodies against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse PKA Abcam, Cambridge, UK) (1:1000 diluted in 5% BSA, both). Secondary horseradish peroxidase (HRP)-conjugated antibodies (1:5000) were added and blots were incubated in a blocking buffer for 1 h at room temperature. Immunoreactive proteins were visualized using the WesternBright^{TM} ECL kit (Advansta, Menlo Park, CA, USA) in chemiluminescence machine (Advansta, Menlo Park, CA, USA).

Determination of extracellular protein kinase A and its autoantibody *in vitro*/*in vivo* mouse model

The presence and level of ECPKA-Ab in murine serum samples were assessed by enzyme-linked immunosorbent assay (ELISA). First, 100 μL of murine serum samples diluted 20-fold in reagent diluent were added to 96-well ELISA plates precoated with mouse recombinant catalytic subunit of PKA (Enzo Life

Sciences Inc, Farmingdale, NY, USA) and incubated for 2 hours at room temperature. The plates were then incubated further with anti-mouse immunoglobulin G:horseradish peroxidase antibodies for 2 hours at room temperature in a dark room. Next, the plates were developed with 3,3',5,5'-tetramethylbenzidine substrate solution for 15 min at room temperature. The reaction was stopped with 100 μL of stop solution. Absorbance was determined at 450 nm using a scanning multi-well spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

The concentration of ECPKA in murine serum samples, conditioned medium of LLC1, and cell homogenization of LLC1 were assessed using mouse protein kinase A catalytic subunit alpha (PRKACA) Sandwich ELISA kit (LSBio, Seattle, WA, USA) following the manufacturer's instructions.

Statistical Analysis

Differences between more than two groups were analyzed using one-way ANOVA, and the differences between two groups were analyzed using the t-test, all of which were in parametric distribution. The Tukey's multiple comparisons test was used as post-hoc analysis after the one-way ANOVA test. All graphs are presented as bar with standard deviation. All the data are shown as the mean ± standard deviation obtained in at least three independent experiments. The statistical analyses were performed using SPSS for Windows version 23 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 7 (GraphPad Software, Inc, La Jolla, CA, USA). A p-value of <0.05 was considered statistically significant.

2.3 Results

PKA concentrations depending on cell number *in vitro*

qRT-PCR revealed that mRNA levels of PRKARIA, PRKARIIB, and PRKACA were not different between LLC1 $4x10^5$ cells and $8x10^5$ cells (Figure 2.2). Western blot revealed that $8x10^5$ cells had higher intracellular PKA levels than $4x10^5$ cells group ($p = 0.0109$, t-test). ELISA revealed that ECPKA level of conditioned medium in $4x10^5$ cells group was 0.0134 ± 0.00131 ng/mL (mean ± SD), and $8x10^5$ cells group was 0.0301 ± 0.00608 ng/mL. There was no statistically significant difference in ECPKA levels in conditioned medium according to cell number ($p = 0.0516$, t-test) but seems to be increased in $8x10^5$ cells group than $4x10^5$ cells group.

ECPKA concentrations in mouse serum depending on tumor size

Serum ECPKA levels were too low and not in detection range of commercial ELISA kit in all control mice and almost tumor bearing mice (Figure 2.3A). Only two mice which was bearing 600 mm^3 tumor and bearing 1000 mm^3 tumor had detectable ECPKA levels in their serum (1.0293 ng/mL and 0.7122 ng/mL, respectively).

ECPKA-Ab concentrations in mouse serum depending on tumor

size

Serum ECPKA-Ab levels had significant difference with tumor size ($p < 0.001$, One-way ANOVA test; Figure 2.3B). Serum ECPKA-Ab level of control mice was 125.44 ± 8.528 ng/mL while serum ECPKA-Ab levels in LLC1 injected mice with tumor size 100 mm^3, 300 mm^3, 600 mm^3, and 1000 mm^3 were 158.83 ± 14.772 ng/mL, 192.84 ± 19.760 ng/mL, 574.01 ± 43.013 ng/mL, and 357.687 ± 53.853 ng/mL, respectively.

PKA concentrations in tumor tissue according to tumor size

After correcting the amount of tissue homogenize with albumin concentration through BCA analysis, PKA level in different sized tumors was analyzed by ELISA. There was no difference in PKA level in the same amount of albumin ($p = 0.928$, One-way ANOVA; Figure 2.3C). PKA levels corrected with 50 ug albumin of tumor size 100 mm^3, 300 mm^3, 600 mm^3, and 1000 mm^3 were 0.32 ± 0.544 ng/mL, 0.32 ± 0.084 ng/mL, 0.33 ± 0.064 ng/mL, and 0.30 ± 0.112 ng/mL. Comparing the PKA levels of whole tumor tissue, the higher PKA level with increased tumor size was evaluated ($p < 0.001$, One-way ANOVA; Figure 2.3D).

2.4 Discussion

In the present study, several experiments were performed to demonstrate my hypothesis that tumor burden increases serum ECPKA and ECPKA-Ab levels in mouse. As a result, positive correlation between both ECPKA and ECPKA-Ab levels versus tumor size was demonstrated by *in vitro* and *in vivo* experiments. Intracellular PKA levels were demonstrated to associated with cancer cell number *in vitro* but not *in vivo* and extracellular PKA levels were seems to associated with cancer cell number and tumor tissue weight both *in vitro* and *in vivo* (Figure 2.2 and Figure 2.3). *In vivo* experiments demonstrated that ECPKA-Ab levels also associated with cancer size.

Firstly, quantitative expression analysis of mRNA levels of PRKARIA, PRKARIIB, and PRKACA was performed *in vitro* study with different cell number of LLC1. Despite our target protein is catalytic subunit Cα of PKA encoded by PRKACA gene, several reports showed that Cα level and PRKACA levels were regulated by regulatory subunits such as RIα encoded by PRKARIA and RIIβ encoded by PRKARIIB (Cho-Chung and Nesterova, 2005, Neary et al., 2004, Cho et al., 2000b, Loilome et al., 2012). In the present study different cell number didn't influence mRNA levels of all three genes (Figure 2.2). It is thought to be a natural result because it is same cancer cell line. But when non-malignancy cell line

transformed to malignant cell line by adding some carcinogens, it is demonstrated that PKAI and RI subunit increased and that regulatory subunit switched from RII to RI isoform (Cho-Chung et al., 1983, Wehner et al., 1981, Tagliaferri et al., 1988, Ciardiello et al., 1990).

PKA levels according to cancer cell number or size were analyzed *in vitro* and *in vivo* study. When calibrated same albumin concentration, intracellular PKA levels were different according to cell number in the present *in vitro* study (Figure 2.2) whereas PKA levels of tissue protein were not different according to cancer size *in vivo* study (Figure 2.3). It might be caused by cell-cell interaction in 2D cell culture which is disadvantages of 2D cell culture system.

ECPKA levels according to tumor size were analyzed *in vitro* and *in vivo* study. ECPKA levels in conditioned media was seemed to be different in cell numbers, but not statistically significant difference ($p = 0.0516$, t-test; Figure 2.2B). It might be caused by too low concentration of PKA to detect in conditioned media. Serum ECPKA levels of control group, 100 mm^3 tumor-bearing group, 300 mm^3 tumor-bearing group, 600 mm^3 tumor-bearing group, and 1000 mm^3 tumor-bearing group were analyzed by ECPKA. ECPKA levels in mouse serum were too low to produce meaningful results, but ECPKA within the detection range was not detected in individuals with small or absent tumors, whereas ECPKA was detected within the detection range in large tumors (Figure 2.3A). It was suggested that ECPKA

levels in serum were increased in large tumors. Moreover, demonstrating consistency of ECPKA-Ab levels with tumor size (Figure 2.3B) could tell that ECPKA level in serum also consistent with tumor size because lots of study demonstrated that autoantibody concentration is positively related with its antigen (Zaenker and Ziman, 2013, Zaenker et al., 2016, Hong et al., 2013, Watanabe et al., 2000, Goodell et al., 2008).

In animal study, serum ECPKA-Ab levels were showed significant consistency with tumor size (Figure 2.3B). This suggests that serum ECPKA-Ab levels could give an information about tumor progression. Compared to ECPKA, ECPKA-Ab is more stable as described in the study conducted by ES CHO et al. and has a much higher amount in serum presented in this study and showed in other studies (Zaenker et al., 2016, Lu et al., 2008, Cho et al., 2000a), which is easier to detect and has the advantage of showing the progress of the tumor. This study showed the possibility of ECPKA-Ab as biomarker for tumor size change such as progression, reduction, or relapse.

Taken together, serum ECPKA-Ab is universal cancer biomarker that could monitor change of tumor size and more useful biomarker than serum ECPKA.

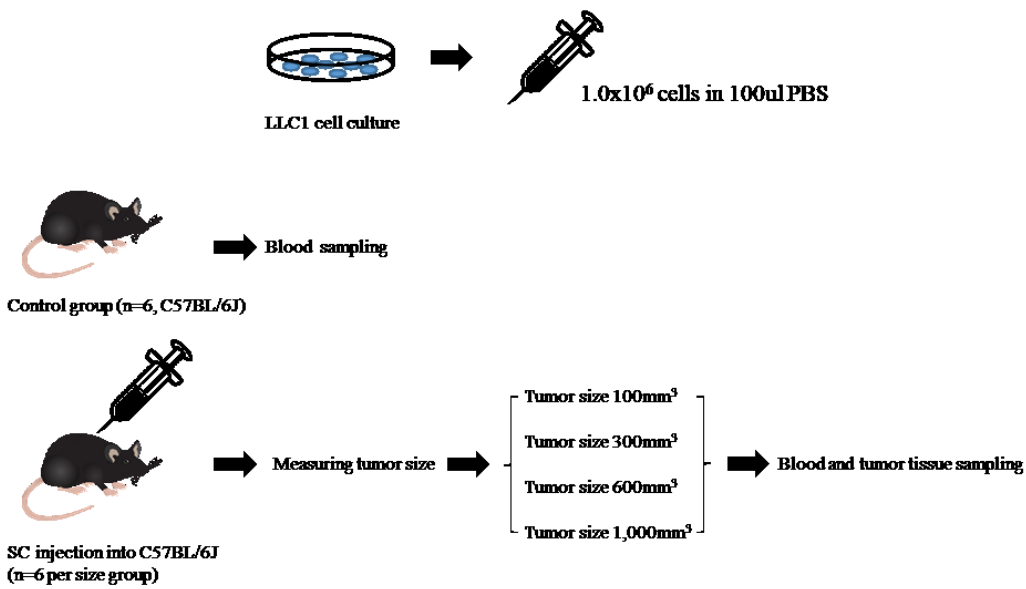

Figure 2.1. Experimental study design in C57BL/6J mice injected with different size of tumor.

Mice were allocated into five experimental groups; Control group (none injected), and experimental groups injected with different size of tumor; 100 mm^3, 300 mm^3, 600 mm^3, and 1000 mm^3. Six mice were included in each group.

Abbreviation; LLC1, Lewis lung carcinoma cell line; SC, subcutaneous; PBS, phosphate buffered saline

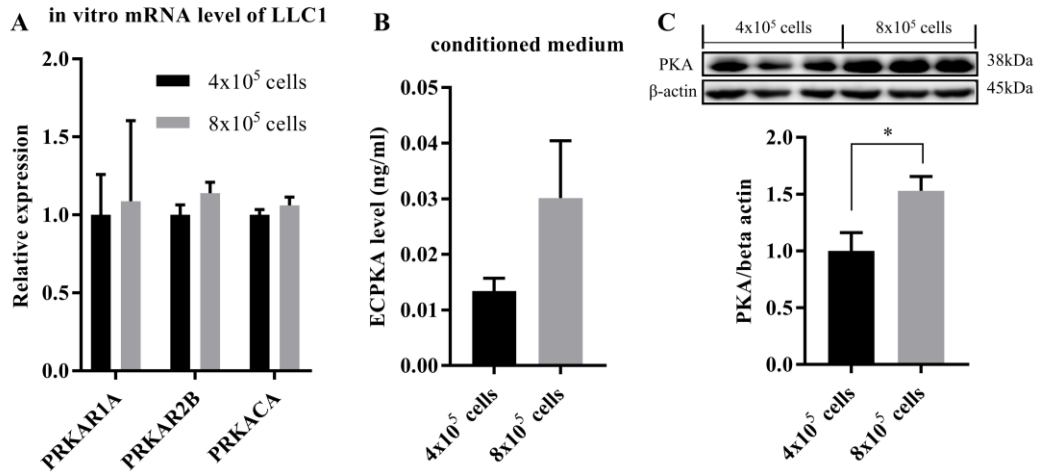

Figure 2.2. mRNA and protein expression levels of protein kinase A on the different cell number.

A) qRT-PCR analysis of PRKARIA, PRKARIIB, and PRKACA which are genes related to PKA excretion. There was no significant difference with different cell number (p=0.8050, p=0.0663, p=0.1758, all t-test). B) ELISA analysis of PKA levels in conditioned medium. There was no significant difference in their PKA levels according to different cell number (p=0.0516, t-test), but mean PKA value of $4x10^5$ cells and $8x10^5$ cells was 0.0134 ng/mL and 0.0301 ng/mL, respectively. C) Western blot analysis of intracellular PKA levels according to different cell number.

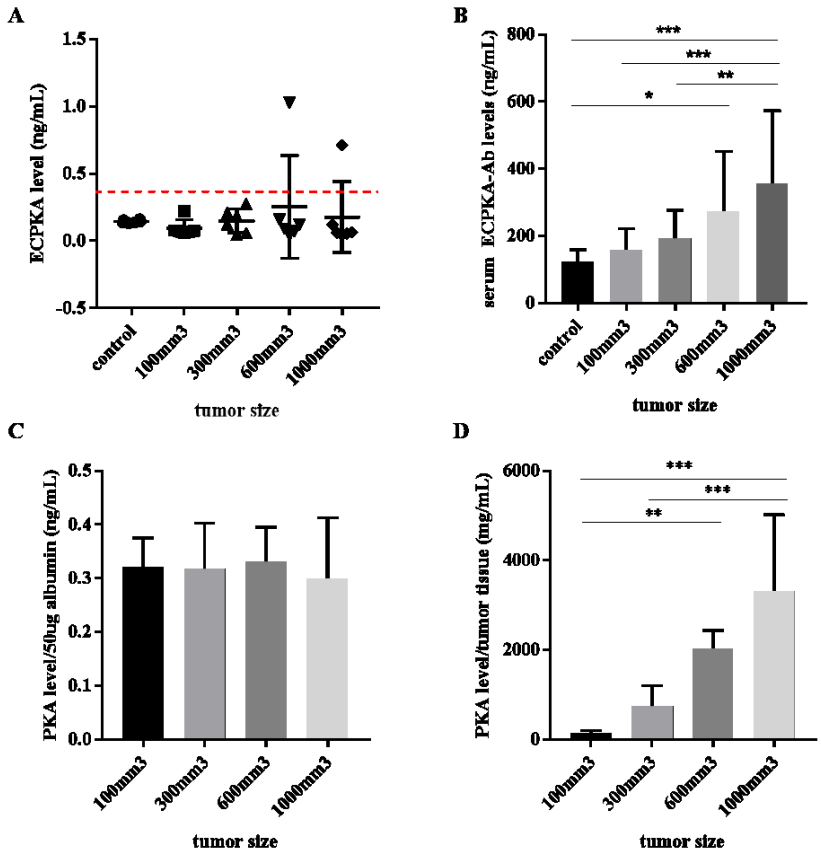

Figure 2.3. PKA levels and ECPKA-Ab levels according to tumor size in mice.

A) serum ECPKA level of C57BL/6J mice with different tumor size. A spot represents a subject and red dotted line means minimum detection concentration of commercial ELISA kit. B) serum ECPKA-Ab level of C57BL/6J mice with different tumor size. There was significant difference in five groups ($p < 0.001$, one-way ANOVA) and post-hoc analysis with Tukey's multiple comparisons test revealed control < 600 mm^3, control < 1000 mm^3, 100 mm^3 < 600 mm^3, and 300 mm^3 < 1000 mm^3 C) PKA levels in different size of tumor tissue calibrated with 50ug albumin. D) PKA levels of total tumor tissue in C57BL/6J mice with different tumor size. There was significant difference in all four groups ($p < 0.001$, one-way ANOVA). Post-hoc analysis with Tukey's multiple comparisons test revealed 100 mm^3 < 600 mm^3, 100 mm^3 < 1000 mm^3, and 300 mm^3 < 1000 mm^3. Asterisk indicates statistically significant P values. *< 0.05, **< 0.01, ***< 0.001

Table 2.1. Primer sequences for quantitative real-time PCR amplification of target genes.

Gene		Primer sets	Product Size (bp)
PRKARIA	F	5' CAG TGA GGA AGA GCG GAG TC 3'	216
	R	5' TCC TCC CTC GAG TCA GTA CG 3'	
PRKARIIB	F	5' AGG TGG TGG ATG TGA TTG GC 3'	193
	R	5' CCA AAG TAC TGG CCC CGA AA 3'	
PRKACA	F	5' AGC AGG AGA GCG TGA AAG AG 3'	184
	R	5' TTC ATG GCG TAG TGG TTC CC 3'	
GAPDH	F	5' TCA TTG ACC TCA ACT ACA TGG TCT A 3'	193
	R	5' ACA CCA GTA GAC TCC ACG ACA TAC T 3'	

Chapter III

Changes in extracellular cyclic AMP-dependent protein kinase A autoantibody levels with respect to cancer progression in dogs with lymphoma and transitional cell carcinoma

Abstract

Extracellular protein kinase A autoantibody (ECPKA-Ab) is present in high concentrations in the serum of dogs with cancer. The aim of this study was to examine whether the serum ECPKA-Ab levels changed with the clinical progression of cancer in dogs. A total 10 dogs with transitional carcinoma (TCC) and 37 dogs with lymphoma were included in this study. We obtained serial sera over a period of 3 years and measured the ECPKA-Ab levels using enzyme-linked immunosorbent assay. The serum ECPKA-Ab levels were found to be increase at 1 to 6 months after the initial sampling in 10 dogs with progressing TCC ($p=0.004$).

In the dogs with lymphoma, the serum ECPKA-Ab levels were significantly different with respect to the level of therapeutic response. Those with progressive disease had higher pre- and post-treatment levels than those in complete remission ($p=0.0010$ and $p=0.0004$, respectively). Relapsed dogs had increased ECPKA-Ab levels when compared to those with a complete remission status ($p=0.0005$, in one-point time data; $p=0.0285$ in serial data of each dog). These findings suggest that serum ECPKA-Ab levels could serve as a predictive biomarker for conventional lymphoma chemotherapy and as a biomarker to monitor the recurrence of lymphoma in remissive dogs or the progression of TCC in dogs.

Keywords: autoantibodies; biomarkers; cyclic AMP-dependent protein kinases; disease progression; dogs; lymphoma

3.1. Introduction

Lymphoma is one of the most common neoplasms, as well as the most common hematopoietic cancer, in dogs (Dorn et al., 1968, Zandvliet, 2016). The World Health Organization (WHO) has issued classification systems for canine lymphoma pertaining to cancer location (generalized, alimentary, thymic, skin, leukemia, and others), stage (I to V), and substage based on systemic signs (a, b) (Owen and Organization, 1980). The most common form is multicentric lymphoma without systemic sickness (WHO substage a) (Ponce et al., 2010). Other classification systems pertain to immunophenotyping of neoplastic clones and grade based on cytologic appearance (low, intermediate, high) (Valli et al., 2011, Zandvliet, 2016, Carter et al., 1986). The mean survival time (MST) for those with untreated lymphoma is 4-6 weeks, though it could be prolonged with systemic therapy, such as chemotherapy, radiotherapy, and immunotherapy (MacEwen et al., 1977, Zandvliet, 2016).

Transitional cell carcinoma (TCC) is the most common urinary bladder cancer of dogs (Mutsaers et al., 2003, Knapp et al., 2000). This invasive cancer is typically located in the urinary bladder trigone region (Mutsaers et al., 2003, Valli et al., 1995). Common clinical symptoms included hematuria, pollakiuria, and dysuria (Knapp, 2006). Female dogs have been shown to be a higher risk for TCC

than male dogs, and neutered dogs have a higher risk than do intact dogs (Knapp et al., 2000). The MST of those with TCC is reported to be less than one year regardless of the administration of different treatments (Stephen, 2013).

Extracellular protein kinase A autoantibody (ECPKA-Ab) is an autoantibody to serum protein kinase A, an essential enzyme for cell proliferation and metabolism, which is leaked from cancer cells (Cho et al., 2000b, Cho et al., 2000a). This autoantibody is present in high concentrations in the serum of humans with various types of cancers (Cho-Chung, 2006, Choi et al., 2006, Nesterova et al., 2006a). My previous study showed elevated concentrations of ECPKA-Ab in dogs with cancer when compared to dogs with healthy, non-neoplastic disease or benign tumors (Reference; Chapter 1). In that study, the receiver operating characteristic curve showed the area under the curve was 0.86 with a specificity of 79.38 and sensitivity of 80.84.

The objective of this study was to evaluate the change in serum ECPKA-Ab levels in dogs with lymphoma and TCC with respect to time and therapeutic response.

3.2 Materials and Methods

Animals and samples

Forty-eight privately owned dogs (10 dogs with transitional cell carcinoma (TCC) and 37 dogs with lymphoma) were included in the study. Serum samples were provided by our university veterinary medical teaching hospital. All of the dogs included in this study underwent a physical examination; urinalysis; blood examination including complete blood counts, serum biochemical profiles, and electrolytes; and diagnostic imaging, such as abdominal sonography, radiography (thoracic and abdominal), or an optional echocardiogram or computed tomography. TCC was diagnosed by cytology and/or histopathology. All of the dogs included this study had progressive TCC with marked clinical progression (e.g. prolonged urination time, increased pain during urination, increased severity of hematuria). Lymphoma was diagnosed by cytology, and immunophenotyping was performed by polymerase chain reaction (PCR). Collected sera were classified as *pre-treatment*, *post-treatment*, and *relapse*. Pre-treatment was defined as the serum obtained from subjects not receiving chemotherapeutic drugs or steroids. Post-treatment was defined as the serum obtained at the end of an entire cycle of the anticancer protocol (samples obtained during the 21-25th week of a short 25-week modified University of Wisconsin Madison CHOP lymphoma protocol) (Garrett et al., 2002) or at the last day of the CHOP protocol if the anticancer protocol was

changed to another protocol (e.g. CCNU or other rescue protocol). The post-treatment therapeutic response was categorized as complete remission (CR), partial remission (PR), static disease (SD), and progressive disease (PD), which were defined by RECIST (response evaluation criteria for solid tumors).[19] Relapse was defined as the serum obtained at the time of recurrence, which determined by the presence of an enlarged lymph node or the appearance of peripheral lymphoblasts after complete remission.

All experiments were approved by and followed the policies and regulations of the Laboratory Animals Institutional Animal Care and Use Committee (SNU-180130-2).

Measurement of autoantibody to ECPKA in canine serum

My previous study reported on the assessment of ECPKA-Ab levels in canine serum samples using enzyme-linked immunosorbent assay (ELISA) (Reference; Chapter 1). Briefly, canine serum samples were added to 96-well ELISA plates pre-coated with canine PKA Cα and incubated with anti-dog immunoglobulin G:horseradish peroxidase antibodies in a dark room. The plates were developed with 3,3',5,5'-tetramethylbenzidine substrate solution. Absorbance was determined at 450 nm using a scanning multi-well spectrophotometer (Molecular Devices, Sunnyvale, CA, USA)

Statistical Analysis

The data were tested for normality using the Shapiro-Wilk test. Differences between more than two groups were analyzed using the Kruskal-Wallis test, and differences between two groups were analyzed using the Mann-Whitney test—all in non-parametric distribution. Dunn's multiple comparisons test was used for post-hoc analysis after the Kruskal-Wallis test. Paired t-test was used to analyze differences in serum ECPKA-Ab levels over time in each dog. Two-way analysis of variance (ANOVA) with repeated measure was used to evaluate differences in the serum ECPKA-Ab levels of each dog in both groups over a period of time, and Sidak's multiple comparisons were used to compare two groups. The correlation of two factors was analyzed using the Spearman correlation test. All graphs are presented as box and whisker plots. All the data are shown as the median and range obtained in at least three independent experiments. The statistical analyses were performed using SPSS for Windows version 23 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 7 (GraphPad Software, Inc., La Jolla, CA, USA). A p-value of <0.05 was considered statistically significant.

3.3 Results

Animal Signalments

Serum samples from 47 dogs with a diagnosis of lymphoma or TCC were included in this study. The signalment data of the dogs are summarized in Table 3.1. The dogs in the pre-treatment group comprised 8 dogs who were denied any treatment, 1 dog with no follow up after the 1st week of the CHOP protocol, and 11 dogs treated with the CHOP protocol. A total of 37 lymphoma dogs were classified according to tumor location: multicentric lymphoma (n = 29), alimentary lymphoma (n = 5), extranodal (n = 2), and cutaneous lymphoma (n =1). They comprised: B cell type (n = 23), T cell type (n = 5), and unknown immunophenotype (n = 9). According to the stage classification, four dogs were stage 1, three dogs were stage 2, six dogs were stage 3, eighteen dogs were stage 4, and six dogs were stage 5. Based on the sub-staging criteria, 25 dogs were substage a and 12 dogs were substage b. A total of 10 dogs with TCC were serially checked for ECPKA-Ab levels in their serum. All of the dogs had undergone therapy with piroxicam, and each case of cancer had progressed with time.

Serum ECPKA-Ab levels with respect to various classification and cancer progression in lymphoma dogs

Serum ECPKA-Ab levels in the pre-treatment lymphoma dogs were

analyzed with regard to location, stage, substage, and immunophenotype. As a result, there was no significant difference (Table 3.2). When the serum ECPKA-Ab levels were measured from dogs undergoing treatment, there were significant differences between values obtained during pre-treatment and complete remission ($p=0.0014$, Mann-Whitney test), between complete remission and progressive disease ($p=0.0039$, Mann-Whitney test), and between complete remission and relapse ($p=0.0005$, Mann-Whitney test; Figure 3.1). When the serum ECPKA-Ab levels were measured to detect the therapeutic response to the CHOP protocol, serial data showed that pre-treatment and post-treatment levels were not significantly different in each subject ($p=0.6367$, two-way ANOVA with RM); however, the CR group had different pre- and post-treatment serum ECPKA levels when compared to that of the SD to PD group ($p=0.0010$ and $p=0.0004$, respectively, Sidak's multiple comparisons test; Figure 3.2A). When 20 pre-treatment sera were classified as showing a therapeutic response to the CHOP protocol, the serum ECPKA-Ab levels of the CR group ($n=5$) were significantly lower than that of the SD to PD group ($n=6$; $p=0.0043$, Mann-Whitney test; Figure 3.2B). The serial data of relapsed lymphoma dogs that previously achieved complete remission are shown in Figure 3.3 ($p=0.0285$, Paired t-test). Overall survival was not correlated with pre-treatment serum ECPKA-Ab levels ($rho=-0.033$, Spearman correlation).

Serum ECPKA-Ab levels with cancer progression in TCC dogs

The serum ECPKA-Ab levels in TCC dogs were analyzed over a period of time. Regardless of the TCC treatment administered, no dogs achieved remission and they all had progressive disease. Our data show the initial serum ECPKA-Ab levels detected and the results of the second sampling performed at 1-6 months following the first assessment. The second samples showed increased levels of ECPKA-Ab when compared to that of the first sample ($p=0.004$, Paired t-test; Figure 3.4). Overall survival was not correlated with the pre-treatment serum ECPKA-Ab levels ($rho=-0.127$, Spearman correlation).

3.4 Discussion

In this study, changes in the serum ECPKA-Ab concentration were observed in TCC dogs exhibiting disease progression over time–a representation of a solid tumor; additionally, serum ECPKA-Ab concentrations in lymphoma dogs was examined—a condition representative of hematopoietic cancer. The difference in the serum ECPKA-Ab levels between the pre-treatment, post-treatment, therapeutic response, and relapse samples were confirmed in dogs with lymphoma; additionally, the serum ECPKA-Ab levels were found to increase with cancer progression in dogs with TCC. Previous studies have shown that ECPKA and ECPKA-Ab levels are significantly elevated in the serum of dogs with cancer (Bhang et al., 2017) (Reference; Chapter 1). This study was conducted to evaluate how the ECPKA-Ab levels change with the clinical progression of disease over time in canines with lymphoma and TCC, which is representative of both hematopoietic and solid cancers.

When comparing the serum samples before and after treatment at one point in time, the ECPKA-Ab levels of lymphoma dogs with good therapeutic effect (complete remission) were significantly lower than those measured before treatment (Figure 3.1); however, in the serial measurement obtained in the same subjects, there was no significant change detected between pre- and post-treatment samples (Figure 2). These results are similar to that of another study that examined

cancer biomarkers in dogs, in which there were no differences in thymidine kinase type 1 (TK1) activity and C-reactive protein (CRP) concentrations regardless of the effect of possible treatment (Selting et al., 2016b).

Rather, the pre- and post-treatment serum ECPKA-Ab levels differed according to the therapeutic response after completing chemotherapy with the CHOP protocol (Figure 3.2). This suggests that the serum ECPKA-Ab levels at the time of diagnosis could be a predictive factor of therapeutic response to the CHOP protocol in lymphoma dogs. In human medicine, many cancer biomarkers, including vascular endothelial growth factor (VEGF), BRCA1, c-kit, epidermal growth factor receptor 1 (EGFR1), estrogen receptor (ER), Her2/neu, K-ras and diffusion magnetic resonance have been proposed as predictive biomarkers (Jain et al., 2009, Hamstra et al., 2007, James et al., 2007, DeMatteo et al., 2009, Sequist et al., 2007, group, 1998, Mass et al., 2005, Mascaux et al., 2005). For example, non-small cell lung cancer patients with resistance to cisplatin-based chemotherapy showed high expression of BRCA, and increased expression of estrogen receptors in breast cancer can predict good response to tamoxifen-based chemotherapy (Cobo Sr et al., 2008, Early breast cancer trialists' collaborative group, 1998, Paik et al., 2006).

Dogs with relapsed lymphoma showed higher ECPKA-Ab levels than did those with remission status (Figure 3.3). Therefore, it is suggested that the ECPKA-

Ab level might be associated with a dramatic increase in tumor volume. Similarly, a study conducted in human patients with Non-Hodgkin's lymphoma showed that the increased ECPKA-Ab levels had decreased with therapeutic response (CR or PR) and that decreased ECPKA-Ab levels increased with the reoccurrence of lymphoma (Choi et al., 2006).

In the case of TCC dogs in the present study, the ECPKA-Ab concentration in the serum was increased after a period of time, possibly due to the progression of solid tumors (Figure 3.4). My previous study showed that the ECPKA concentration was increased with elevated cancer cell numbers and tumor volumes (Chapter 2). A previous human study showed that tumor size was a major prognostic factor in cutaneous T-cell lymphoma (Martí et al., 1991). Another study introduced a mathematical model to estimate tumor size based on cancer biomarker assays, which included CA125 and prostate specific antigen (Lutz et al., 2008).

The serum ECPKA-Ab levels were also analyzed using various classification criteria for tumor location, stage, and substage, all of which were derived from the World Health Organization's clinical staging system for lymphoma in domestic animals (Owen and Organization, 1980), immunophenotype. There was no statistical difference in the serum ECPKA-Ab concentration according to this classification method. However, with regard to immunophenotype classification, ECPKA-Ab levels were found to be elevated in those with T-cell type

lymphoma; a statistically significant difference might be observed if there was a larger sample of T-cell lymphoma dogs. In a UK study on canine lymphoma, TK1 activity was found to be higher in dogs with B-cell lymphoma than in those with T-cell type, and it was shown that TK1 activity was not associated with cancer stage, substage, duration of first remission, or survival (Elliott et al., 2013). In the present study, there is a limitation in not analyzing the difference in serum ECPKA-Ab levels with respect to the mitotic index or grade of cancer.

In the present study, there was no correlation between the pre-treatment serum ECPKA-Ab levels and the overall survival in both the TCC and lymphoma dogs. In human lymphoma, several biomarkers, which could be predictive outcome, were discovered. High expression of Ki-67 and CD5 levels was demonstrated to be associated with a poor prognosis in lymphoma patients (Lossos and Morgensztern, 2006, Ennishi et al., 2008, Räty et al., 2002).

There were several limitations in this study. First, only small-breed dogs were included in this study, and the study's sample size was small. We hope to conduct further study with large-breed dogs and with a larger study group.

Altogether, my findings suggest that the serum ECPKA-Ab levels could be a predictive biomarker of therapeutic response to the CHOP protocol in lymphoma dogs, and it could be used as a monitoring biomarker for relapse lymphoma dogs with remission status. Furthermore, the serum ECPKA-Ab levels might be

positively correlated with solid tumor size, such as in TCC. Therefore, it could potentially be used as a biomarker to monitor the progression of solid tumors. This study is the first to examine the potential clinical application of serum ECPKA-Ab as a predictive biomarker to monitor the progression of cancer in dogs.

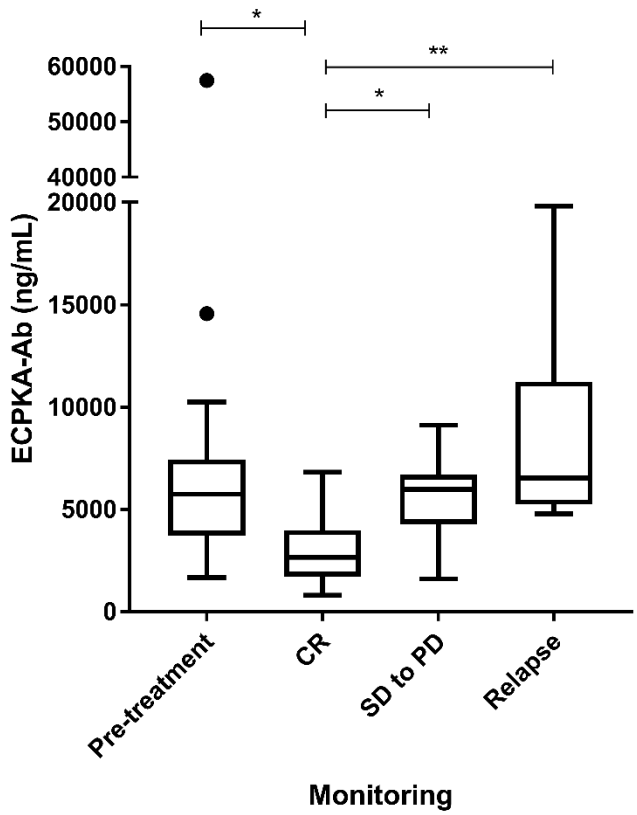

Figure 3.1. Serum ECPKA-Ab levels in lymphoma dogs according to disease status. Serum ECPKA-Ab levels in lymphoma dogs were classified according to the sampling status: pre-treatment (n = 20), CR (n = 13), SD to PD (n = 11), and relapse (n = 7).

CR, complete remission; SD, static disease; PD, progressive disease

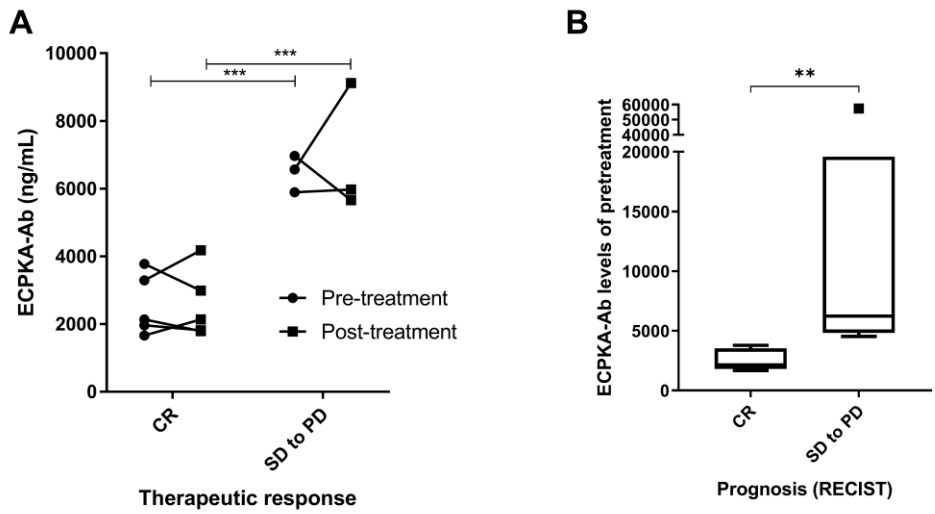

Figure 3.2. Serum ECPKA-Ab levels of pre-treatment versus post-treatment in respond to CHOP in lymphoma dogs.

All dogs were treated with CHOP protocol. A) Serial change of serum ECPKA-Ab levels in respective dogs (total dogs, n = 8). Left dot represents pre-treatment serum ECPKA-Ab levels, and the right dots represents post-treatment serum ECPKA-Ab levels. B) Pre-treatment serum ECPKA-Ab levels of CR dogs (n = 5) and SD to PD dogs (n = 6).

CR, complete remission; SD, static disease; PD, progressive disease

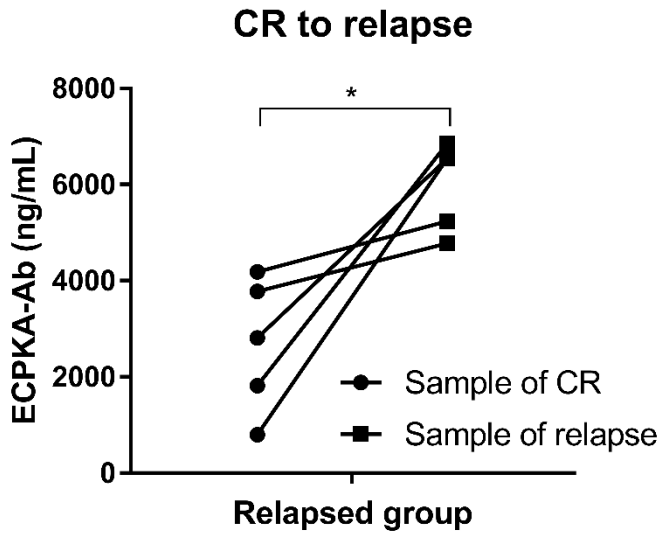

Figure 3.3. Serum ECPKA-Ab levels of relapsed lymphoma dogs with respect to post-treatment status.

Serial change of serum ECPKA-Ab levels of respective relapsed lymphoma dog (n=5).

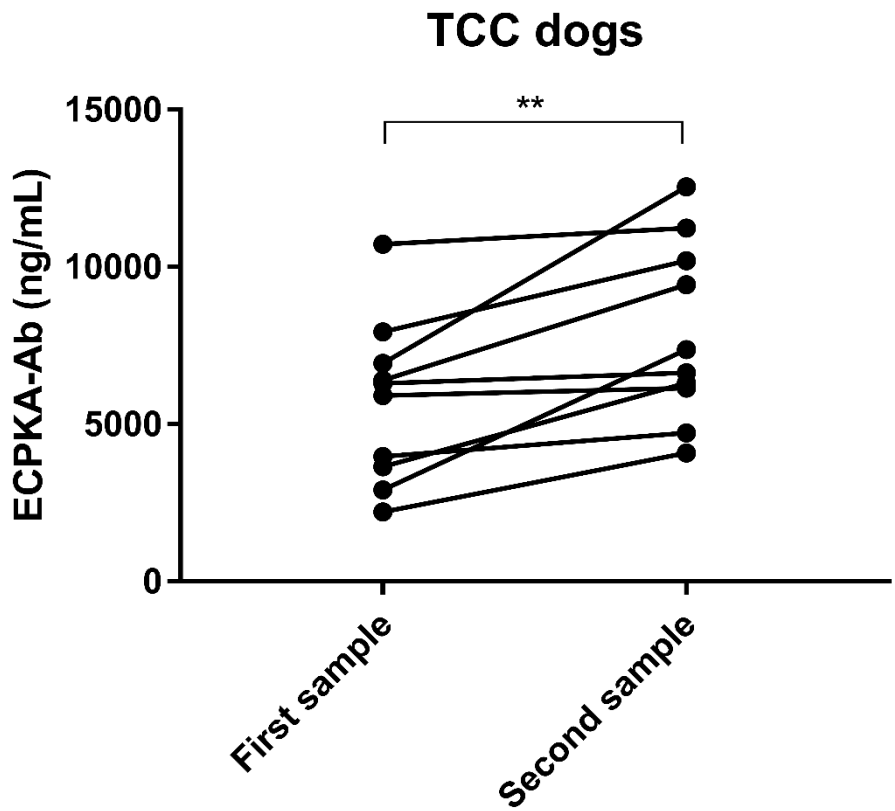

Figure 3.4. Serum ECPKA-Ab levels in TCC dogs with clinical progression. Changes in serum ECPKA-Ab levels sampled at intervals between 1 and 6 months in TCC dogs (n=9).

Table 3.1. Signalment of dogs included in this study.

	Lymphoma	Transitional cell carcinoma
N	37	10
Age median (range)	11.00 (2.00-17.00)	13.00 (12.00-15.00)
Sex (N)	CM (15), F (4), M (1), SF (17)	CM (2), F (2), M (0), SF (6)
Breed (N)	Shih-tzu (15), Maltese (6), Mongrel (5), Cocker Spaniel (2), Schnauzer (2), Yorkshire terrier (2), Other (5)	Maltese (6), Shih-tzu (2), Mongrel (1), White terrier (1)
Pre-treatment (N)	20	3
Post-treatment (N)	CR (13), PD (9), SD (2)	PD (10)
Relapse (N)	7	-

N, number; CM, castrated male; F, female; M, male; SF, spayed female; CR, complete remission; PD, progressive disease; SD, static disease

Table 3.2. Median serum ECPKA-Ab levels according to various classification of canine lymphoma.

	Classification					p-value
Location	Multicentric		Alimentary	Extranodal		
N	14		4	2		
ECPKA-Ab (ng/ml)	5815.0		4837.5	6172.5		0.727
Stage	1	2	3	4	5	
N	3	2	6	7	2	
ECPKA-Ab (ng/ml)	4475.0	4837.5	6272.5	6540.0	3337.5	0.551
Substage	a		b			
N	13		7			
ECPKA-Ab (ng/ml)	4920.0		6970.0			0.275
Immunophenotyping	B		T			
N	23		5			
ECPKA-Ab (ng/ml)	4535.0		6055.0			0.103

ECPKA-Ab, extracellular protein kinase A autoantibody; N, number

GENERAL CONCLUSIONS

Cancer biomarker could give information about the risk, prognosis, progression, and presence of cancer, and the therapeutic response of cancer patients to specific anti-cancer drugs. There is an increased need for cancer biomarkers in veterinary medicine, as the diagnosis rate of cancers continues to increase and the mortality of cancer is highest in adult dogs. It was hypothesized that ECPKA-Ab could be a universal cancer biomarker in dogs, like the case in humans. To demonstrate this hypothesis, several experiments were conducted and conclusions are as follows:

1) ECPKA-Ab is present in high levels in the sera of dogs with various cancers including malignant melanoma, lymphoma, hepatocellular carcinoma, transitional cell carcinoma, hemangiosarcoma, pulmonary adenocarcinoma, malignant melanoma. On the other hand, ECPKA-Ab is present in low levels in the sera of healthy dogs and dogs with benign tumor and non-tumor disease. Considering the receiver operating characteristic curves (area under the receiver operating characteristic curves: 0.86), it is considered to be a good biomarker for cancer. High CRP levels were also present in the sera of dogs with cancer. Combining the ECPKA-Ab and

CRP parameters, a new neoplastic index was developed. The neoplastic index had higher sensitivity, specificity, and accuracy than the ECPKA-Ab measurement alone, but there was little difference between them. It is proposed that ECPKA-Ab and the neoplastic index could be a universal cancer marker in dogs and CRP could be a good adjuvant for developing cancer diagnostic tool.

2) In the *in vitro* study with LLC1 cell lines, increased cell number was related to increased protein levels of intracellular and extracellular PKA concentrations, but not mRNA levels. In the *in vivo* study with cancer-bearing mouse model, serum ECPKA levels were undetectable in almost mice of all groups; control group, tumor100 mm^3-bearing group, tumor300 mm^3-bearing group, tumor600 mm^3-bearing group, and tumor1000 mm^3-bearing group. On the other hand, serum ECPKA-Ab levels were enough to detect and increased with enlarging tumor size, suggesting that ECPKA-Ab could be used for assessing whether the cancer size increases or decreases, and whether the cancer has progressed, reduced, or relapsed. Moreover, this study demonstrated that ECPKA-Ab was easier to detect than ECPKA like as other autoantibody of tumor-associated antigen exist high levels in serum despite low levels of the corresponding antigen.

3) Serum samples of lymphoma-affected dogs were categorized with regards to their treatment statuses as pre-treatment, post-treatment, and relapse; the serum ECPKA-Ab levels were significantly different between these categories. Moreover, pre-treatment samples were categorized by therapeutic response to conventional chemotherapy; dogs in remission had significantly lower serum ECPKA-Ab levels before the start of chemotherapy than dogs with progressive disease, suggesting that the serum ECPKA-Ab level could be a predictive marker for conventional chemotherapy in lymphoma-affected dogs.

4) Serum ECPKA-Ab levels, which was continuously measured according to the treatment status as pre-treatment, post-treatment, and relapse, was analyzed in the same subject of lymphoma-affected dogs; serum ECPKA-Ab levels increased with relapse in lymphoma-complete remissive dogs. Considering this, ECPKA-Ab could be used for risk assessment of relapse or monitoring the remission status in lymphoma-affected dogs.

5) All dogs with transitional cell carcinoma had progressive disease despite of

taking piroxicam. Serum samples were serially collected from the dogs; the serum obtained 1 -6 months after the start had higher ECPKA-Ab levels than that at the start. It is considered that progressive solid tumors increase the serum ECPKA-Ab levels. It is proposed that serum ECPKA-Ab could help monitor cancer progression of solid tumors.

Collectively, it is proposed that ECPKA-Ab could be a universal cancer biomarker in dogs, with potential for use in several clinical applications, including screening for occult cancer in dogs with subclinical disease, and monitoring cancer progression of certain cancers and therapeutic response to chemotherapeutic drugs on specific cancer, for which it serves as a predictive marker. Further studies about combining ECPKA-Ab and other biomarkers could improve the accuracy of diagnosing cancers in dog.

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국 문 초 록

개의 악성종양 바이오마커로서의 세포외단백질인산화효소 A에 대한 자가항체

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암은 개의 사망률 1위 질환이며, 진단 기술의 발달로 인해 개의 암 유병율은 점점 높아지고 있다. 암환자에서 생존기간을 늘리고 삶의 질을 증가시키기 위해서 암을 조기에 진단하여 적절한 치료가 수행되어

야 한다. 암 바이오마커는 암을 조기에 확인할 수 있는 하나의 방법으로써, 암 바이오마커의 안전성, 편리성, 임상에서 적용 가능한 다양한 잠재적 가능성 때문에 암 바이오마커에 대한 관심이 높아지고 있다. 임상에서 암 바이오마커는 환자가 암에 걸릴 수 있는 잠재적 위험이나 암의 이환 가능성을 평가할 수 있으며, 암의 진단, 진행, 치료 상태를 관찰하는 데 이용될 수 있다. 또한, 특정 약물에의 치료 반응을 예측할 수 있으며, 암의 예후에 대한 정보를 알려줄 수 있다. 사람에서는 이미 다양한 암 바이오마커가 연구되었고, 그 중 몇 가지는 임상에서 사용이 되고 있다. 세포외단백질인산화효소 A의 자가항체는 인의에서 보편적 악성종양 바이오마커로 사용될 수 있음이 증명되었지만, 수의학에서는 악성종양을 가진 개 환자에서의 세포외단백질인산화효소 A 자가항체에 대한 연구가 전무한 실정이다. 따라서, 혈청에 존재하는 세포외단백질인산화효소 A 자가항체가 개에서도 악성종양의 바이오마커로 이용될 수 있을 것이라는 가설을 세우고 연속적인 연구를 진행하게 되었다.

먼저, 악성종양을 가진 개에서 세포외단백질인산화효소 A 자가항체와 급성 염증 단백질인 C 반응성 단백질이 혈청 중에서 높게 존재하는지의 여부를 확인하는 연구를 진행하였다. 서울대학교 수의과대학 부속동물병원과 국내 지역병원에 내원한 총 487 마리의 개의 혈청을

실험에 이용하였다. 총 487 마리의 개는 123마리의 건강한 개와 155 마리의 비종양성 질환의 개, 42마리의 양성 종양을 가진 개, 167마리의 악성 종양을 가진 개로 구성되었다. 혈청에서의 세포외단백질인산화효소 A 자가항체와 C 반응성 단백질의 양은 ELISA 방법을 이용해 검출하였다. 세포외단백질인산화효소 A 자가항체와 C 반응성 단백질을 각각 변수로 한 다중회귀분석을 통해 새로운 지표인 Neoplastic index (NI)를 만들었다. 악성종양을 가진 개에서는 악성종양이 없는 개에 비해 세포외단백질인산화효소 A 자가항체와 NI가 모두 유의적으로 높게 확인되었다. 인자의 악성종양 구별 능력 정도를 확인하는 수신자 조작특성 (receiver operating characteristic)은 세포외단백질인산화효소 A 자가항체와 NI에서 곡선아래면적이 각각 0.86, 0.89로 높게 나타났다. NI 지수는 세포외단백질인산화효소 A 자가항체에 비해 암을 진단하는데 있어 민감도, 특이도, 정확도가 약간씩 높은 것으로 확인되었다. 결론적으로 세포외단백질인산화효소 A 자가항체와 NI 지수는 모두 개에서 보편적 악성종양 바이오마커로 이용될 수 있을 것으로 판단되었다.

다음으로, 세포외단백질인산화효소 A와 세포외단백질인산화효소 A 자가항체가 종양의 크기에 의존적인지의 여부를 확인하기 위해 Lewis lung carcinoma (LLC1) 세포주를 이용하여 생체 외 (*in vitro*) 검

사와 생체 내 (*in vivo*) 검사를 진행하였다. 생체 외 검사에서는 LLC1 세포의 양을 다르게 배양하였을 때 세포 내에서 단백질인산화효소 A의 유출과 관계 있는 PRKARⅠA, PRKARⅡB, PRKACA 세 개의 유전자 발현 정도의 차이를 확인하기 위하여 qRT-PCR 검사를 진행하였으며, 세포 내 단백질인산화효소 A의 양은 western blot 검사법을 통해 확인하였다. 세포 외 단백질인산화효소 A의 양은 배양 배지를 이용해 ELISA를 진행하여 분석하였다. 생체 내 연구는 C57BL/6J 마우스를 이용하여 진행하였으며, 종양의 크기에 따른 단백질인산화효소 A와 자가항체의 양을 ELISA를 이용하여 분석하였다. C57BL/6J 마우스는 총 다섯 개의 그룹 (대조군, 종양 100 mm^3, 종양 300 mm^3, 종양 600 mm^3, 종양 1000 mm^3)으로 구분되었다. 그 결과, 세포외단백질인산화효소 A는 혈청 중에서 검출할 수 없는 정도로 미량 존재하는 것이 확인된 반면, 세포외단백질인산화효소 A 자가항체는 혈청 중에서 매우 높은 농도로 존재하며, 종양의 크기가 증가할수록 혈청 내 농도가 증가하는 것이 확인되었다. 이를 통해 세포외단백질인산화효소 A 자가항체가 세포외단백질인산화효소 A 자체보다 검출이 용이하며, 종양의 크기에 비례하여 증가한다는 것을 확인하였다.

마지막으로, 자연적으로 발생한 림프종과 방광이행상피암종의

개에서 질병이 진행에 따라 혈청 중 세포외단백질인산화효소 A 자가항체의 양이 어떻게 변화하는지 분석하였다. 개에서 가장 유병율이 높은 악성종양으로 혈액암과 고형암의 대표적 종양인 림프종과 방광이행상피암종을 각각 선정하였다. 혈청 샘플은 치료 여부와 시기에 따라서 치료 전, 치료 후, 재발로 구분하였으며, 치료 후 샘플은 치료 반응에 따라 병의 완전한 차도, 부분적인 차도, 변화 없음, 진행성 질병으로 구분하였다. 혈청 중 세포외단백질인산화효소 A 자가항체의 양은 치료 여부와 시기에 따라 다르게 나타났으며, 일반적인 개 림프종 치료요법인 CHOP protocol로 치료를 하였을 때, 치료 전 샘플에서의 세포외단백질인산화효소 A 자가항체의 농도가 치료 반응이 좋은 개체는 치료에 반응이 없는 개체에 비해 유의적으로 낮은 것이 확인되었다. 방광이행상피암종을 앓고 있는 개들은 모두 piroxicam을 적용 중이었으나 질병이 악화되었고, 질병이 악화됨에 따라 혈청 내 세포외단백질인산화효소 A 자가항체의 농도가 증가하는 것을 확인할 수 있었다. 따라서 혈청 세포외단백질인산화효소 A 자가항체의 양은 림프종과 방광이행상피암종을 앓고 있는 개에서 치료반응을 예측하는 바이오마커가 될 수 있을 것으로 추정한다.

종합해 보았을 때, 혈청 중에 존재하는 세포외단백질인산화효소

A 자가항체는 개에서 보편적 악성종양 바이오마커가 될 수 있을 것으로 생각되며, 암의 존재에 대한 정보뿐만 아니라 일부 종양에서의 특정 항암제에 대한 치료 반응 예측, 고형암의 진행 상황 관찰에 대한 정보까지 제공해주는 바이오마커로 기능할 수 있을 것으로 생각된다.

주요어: 자가항체 / 바이오마커 / 악성종양 / 개 / 세포외단백질인산화효소 A

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