Studies on p16-pRb pathway in canine lymphoma cells

（犬リンパ腫細胞を用いた p16-pRB 経路に関する研究）

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March 2023

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GENERAL INTRODUCTION

The lymphomas (malignant lymphoma or lymphosarcoma) are categorized as the most common hematopoietic tumor and are defined as a proliferation of malignant lymphoid cells affecting primarily the lymph nodes or solid visceral organs, such as the liver and spleen [Vail, 2010, Vail *et al*., 2013]. Lymphoma is the most common hematological malignancy in dogs [Elvers *et al*., 2015, Valli *et al*., 2013]. Since dogs spontaneously develop lymphoma [Elvers *et al*., 2015], canine lymphoma is being a promising spontaneous large-animal model for human lymphoma [Zandvliet, 2016]. Canine lymphoma resembles human lymphoma in many important ways, including characteristic translocations, molecular abnormalities and similar therapeutic responses to chemotherapy, radiation, and newer targeted therapies [Richards *et al*., 2015].

Many forms of lymphomas have specific genetic abnormalities. These abnormalities may be used in both as diagnostic and as prognostic factors [Kluin *et al*., 2011]. Although several effective biomarkers have been identified, clinical cytology, biopsy, medical imaging, and ultrasound are still the standard methods for the early detection of lymphoma in the clinic [Elvers *et al*., 2015]. Molecular techniques can be used to establish a diagnosis of lymphoma or to further characterize the tumor after the initial diagnosis is made. These include (immuno)histochemical and polymerase chain reaction (PCR) techniques [Withrow *et al*., 2013]. For example, the immunophenotype (B-cell versus T-cell) [Vail *et al*., 1997, Wilkerson *et al*., 2005], proliferation rate [Ki67], proliferating cell nuclear antigen [PCNA] expression, argyrophilic nuclear organizer regions [AgNOR] [Bauer *et al*., 2007, Fournel-Fleury *et al*., 1997, Vail *et al*., 1997, Hung *et al*., 2000, Phillips *et al*., 2000, Vajdovich *et al*., 2004], and clonality (PCR for antigen receptor gene rearrangement [PARR]) [Burnett *et al*., 2003] on the tumor can be determined. However, at present, only immunophenotype and PARR clonality assays are routinely used in dogs to inform clinical decision-making [Vail *et al*., 2013].

Molecular genetic analyses in the field of cancer have now provided a sensitive and flexible approach to the provision number of diagnostic possibilities, including sequence analysis, detection of base mutations, demonstration of chimeric gene products, and the determination of the presence or absence of altered DNA related to cancers [Cotter, 1996]. For example, as the PCR-based methods in canine lymphoma studies, the altered p16 gene expression has been examined using semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time PCR [Fosmire *et al.*, 2007, Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a, Fujiwara-Igarashi *et al.*, 2014b]. Although many analyses of altered DNA are currently being applied to canine lymphoma [Elvers *et al*., 2015], the information on molecular biology is still limited.

The p16 (also known as CDKN2A) is an inhibitor of cyclin-dependent kinases 4 and 6 (CDK4/6) which is involved in the negative regulation of the cell cycle. It slows down the cell cycle by prohibiting progression from G1 phase to S phase. Otherwise, CDK4/6 binds cyclin D1 and forms an active protein complex that phosphorylates retinoblastoma protein (pRb) [Rayess *et al*., 2012]. The p16 protein inhibits CDK4/6, keeps hypophosphorylated of pRb, and prevents cell cycle progression [Peurala *et al*., 2013].

Currently, p16 is considered as a tumor suppressor gene because of its physiological role and downregulated expression in a large number of tumors [Romagosa *et al*., 2011]. This is connected to the processes of tumor oncogenesis, and senescence, methylation, mutation, or deletion of p16 results in the downregulation of the gene and leads to cell cycle progression in cancer. A consequence of p16 inactivation is elevated CDK4/6 activity resulting in hyperphosphorylation of pRb [Ohtani *et al*., 2004]. Loss of p16 leads to disruption of the p16-cyclin D1-CDK4/6-Rb pathway, resulting in cell cycle progression [Peurala *et al*., 2013]. The p16-Rb tumor suppressor pathway is abrogated in many types of human cancers, for example in 98% of pancreatic carcinomas through the inactivation of the p16 gene [Schutte *et al*, 1997],

in 92% of melanoma cell lines through the aberration of p16 or CDK4 [Castellano *et al*, 1997], and in 42.7% of diffuse large B-cell lymphoma (DLBCL) cases, respectively, through the deletion or methylation of the p16 gene [Guney *et al*, 2012].

Inactivation of p16 gene has been reported in the canine lymphoma cell lines (CLBL-1, GL-1, Nody-1, Ema, and UL-1), and hypermethylation of the p16 gene has also been identified in the CLBL-1, GL-1, and UL-1 cell lines [Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a] and in canine lymphoma cells obtained from naturally occurring clinical cases [Fujiwara-Igarashi *et al.*, 2014b]. Furthermore, deletion of the p16 gene and pRb phosphorylation reached 100% [Fosmire *et al.*, 2007] in canine high-grade T-cell non-Hodgkin lymphoma (NHL) cases and correlated with poor prognosis [Modiano *et al.*, 2007]. It is suggesting that the p16-pRb pathway is a prognostically valuable parameter for canine lymphoma. However, in these previous studies, p16 protein expression was not examined, as the appropriate antibody that can detect canine p16 protein was not identified until 2017 [Murphy *et al.*, 2017]. Moreover, simultaneous analyses of the p16 gene, its protein expression, and its correlation with Rb protein phosphorylation have not been performed in canine cancers.

Cyclin-dependent kinases 4 and 6 inhibitors (CDK4/6i), namely palbociclib and abemaciclib, are drugs that currently approved for the treatment of breast cancers in humans, in combination with other therapeutics by Food and Drug Administration (FDA) and European Medicines Agency (EMA) [O'Leary *et al*., 2016, Parylo *et al*., 2019]. Their inhibition affects cancer cell proliferation, blocking the progression from G1 to S phase, and may inhibit the metastatic potential of cancer cells, reducing their migration and angiogenesis [Kollmann *et al*., 2019].

In human cancer cell lines, it has been reported that CDK4/6i, palbociclib, can effectively inhibit the proliferation of the tumor cells that lose endogenous inhibition of CDK4/6 because of p16 gene methylation or deletion [Li *et al.*, 2019]. In human melanoma,

glioblastoma, and ovarian cancer cell lines have implicated the loss of p16 in palbociclib sensitivity [Chen *et al*., 2012, Konecny *et al*., 2011, Wiedemeyer *et al*., 2010, Young *et al*., 2014]. Abemaciclib has been shown to reduce the phosphorylated pRb in colorectal cancer and melanoma xenografts [Gelbert *et al*., 2014, Tate *et al*., 2014]. In dogs, CDK4/6i, palbociclib, has antitumor effects on canine mammary tumor cells [Schoos *et al*., 2019] and could potentially be used as a new anti-cancer treatment for canine melanoma [Bongiovani *et al*., 2021]. In contrast, to the best of my knowledge, there is no data available simultaneously analyzing the expression of p16 protein and phosphorylated pRb in dogs treated with CDK4/6i.

From these backgrounds, a series of the present studies were carried out to clarify the direct relationship of the p16 gene and its protein expression with pRb phosphorylation, and p16/pRb pathway in canine lymphoma cell lines treated with CDK4/6 inhibitors. In chapter 1, I verified the correlation between the inactivation of the p16 gene and its protein expression with the methylation of that gene, as well as hyperphosphorylation of the Rb protein. In chapter 2, I explored the correlation between CDK4/6 inhibitors (palbociclib and abemaciclib) sensitivity with expression levels of the p16 protein and phosphorylated pRb in canine lymphoma cell lines.

Chapter 1

Simultaneous analysis of the p16 gene and protein in canine lymphoma cells and their correlation with pRb phosphorylation

SUMMARY

Lymphoma is one of the most frequently diagnosed malignancies in dogs. The most common epigenetic alteration is gene methylation. Methylation of the p16 gene leads to decreased expression of its protein. The p16 protein inhibits the activity of cyclin-dependent kinase, as a negative control of the cell cycle to prevent phosphorylation of the retinoblastoma (pRb) protein. The methylation of the p16 gene has been reported in canine lymphomas, however, p16 protein expression has not been examined in previous studies. In this study, the gene and protein expression of p16, and phosphorylation of pRb, were examined simultaneously in canine lymphoma/leukemia cell lines treated with or without a demethylation drug *in vitro*. I identified the hypermethylation of the p16 gene, the decreased expression of p16 protein and the hyperphosphorylation of pRb in four out of eight cell lines. Furthermore, I revealed that the expression of the p16 protein was more stable than that of the p16 gene and more closely related to the phosphorylation of pRb. In conclusion, the p16 protein expression is suggested as a promising biomarker for canine lymphoma cells, and the p16–pRb pathway could be a target for the better treatment of canine lymphomas.

INTRODUCTION

Lymphoma is one of the most frequently diagnosed malignancies in dogs and is comparable to human lymphoma [Valli *et al*., 2013, Zandvliet, 2016]. The incidence rate of canine lymphomas is estimated at 20–100 cases per 100,000 dogs [Zandvliet, 2016]. Life expectancy varies depending on the classification, such as grade or immunophenotype [Vail, 2010]. In a study of 608 cases of canine malignant lymphoma, 24.5% and 75.5% were classified as low- and high-grade lymphomas, respectively [Ponce *et al.*, 2010]. Low-grade lymphomas usually show an indolent nature and tend to survive longer than high-grade lymphomas. Canine B-cell lymphomas have a high prevalence (97/123; 79.9%) [Vezzzali *et al.*, 2009]. Moreover, molecular immunophenotypes present in the dogs under study were B-cell lymphomas (753/1226; 61.4%) and T-cell lymphomas (473/1226; 38.6%) [Modiano *et al.*, 2005]. Remission and survival times in high-grade T-cell lymphomas have been shown to be shorter than those in high-grade B-cell lymphomas [Valli *et al*., 2013, Zandvliet, 2016].

Many forms of human lymphoma have specific genetic abnormalities. These abnormalities can be used as diagnostic and prognostic factors [Kluin *et al*., 2011]. The translocation of t(8;14) (q24;q32), which results in the rearrangement of the myc proto-oncogene with an immunoglobulin heavy chain, is an important genetic characteristic of Burkitt lymphoma [Boxer *et al*., 2001, Kos *et al.*, 2021, Ott *et al.*, 2013]. In follicular lymphoma (FL), a translocation of t(14;18)(q32;q21), which results in an augmented expression of the BCL2 gene, is often used for diagnostic purposes [Einerson *et* al., 2005, Kos *et al.*, 2021, Matsumoto *et al.*, 2004]. Overexpression of the cyclin D1 protein, a regulator of the early phases of the cell cycle through inactivation of the tumor suppressor retinoblastoma protein (pRb), plays an important role in the pathogenesis of mantle cell lymphoma (MCL) [Kos *et al.*, 2021, Quintanilla-Martinez *et al.*, 2003]. Moreover, aberrant epigenetic alterations, including the regulation of the expression of genes and signal transduction, play an important

role in the pathogenesis and development of lymphoma. The most common epigenetic alterations are DNA methylation and histone modification [Zhang *et al*., 2020]. Although similar studies are currently being conducted on canine lymphomas [Elvers *et al.,* 2015], the information on their molecular biology is still limited.

Cyclin-dependent kinase inhibitor p16 (CDKN2A) primarily functions as a negative regulator of cell cycle progression via the pRb pathway. The p16 protein inhibits the activity of cyclin-dependent kinase (CDK), prevents the phosphorylation of pRb, and thus plays a critical role in cell cycle arrest [Fosmire *et al.*, 2007].

In human lymphomas, several studies have demonstrated that hypermethylation of the p16 promoter region leads to the loss of p16 gene expression [Zhao *et al*., 2016]. In human Burkitt lymphoma, p16 methylation was detectable in 72% of cases, whereas p16 promoter methylation was detected in 80% of patients with stage III/IV [Robaina *et al.*, 2015]. In human diffuse large B-cell lymphoma (DLBCL), p16 is one of the most frequently deleted driver genes [Fan *et al.*, 2020], and its genetic alterations are associated with significantly poorer survival [Reddy *et al*., 2017]. Inactivation of p16 has been reported in canine lymphomas. Decreased expression of the p16 gene has been reported in the CLBL-1, GL-1, Nody-1, Ema, and UL-1 canine lymphoma cell lines, and hypermethylation of the p16 gene has also been identified in the CLBL-1, GL-1, and UL-1 cell lines [Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a]. Moreover, decreased expression of the p16 gene (B-cells: 32/49, 65%; T-cells 9/13, 69%), as well as its hypermethylation (B-cells: 13/55, 24%; T-cells 7/13, 54%) have been reported in canine lymphoma cells obtained from naturally occurring clinical cases [Fujiwara-Igarashi *et al.*, 2014b]. In addition, in canine high-grade T-cell non-Hodgkin lymphoma (NHL) cases, deletion of the p16 gene and pRb phosphorylation reached 100% [Fosmire *et al.*, 2007], suggesting that the p16 gene deletion and pRb phosphorylation are prognostically valuable parameters for canine NHL. Moreover, in canine T-cell lymphomas, p16 inactivation through

loss of chromosome 11, in which the p16 gene is located, or deletion of the p16 gene were found to be correlated with poor prognosis [Modiano *et al*., 2007]. However, in these previous studies, p16 protein expression was not examined, as the appropriate antibody that can detect canine p16 protein was not identified until 2017 [Murphy *et al*., 2017]. To the best of our knowledge, simultaneous analyses of the p16 gene, its protein expression, and their correlation with Rb protein phosphorylation have not been performed in canine cancers. In this study, to clarify the direct relationship between the inactivation of the p16 gene and its protein expression, as well as pRb activation, I (1) identified whether decreased p16 gene expression was related to the absence of that protein expression; (2) determine if the decrease in p16 gene and protein expression was associated with the methylation of that gene; (3) identified if any decrease was related to the hyperphosphorylation of the Rb protein; and (4) determined if the removal of the methylation of the p16 gene was related to the decrease in phosphorylation of the Rb protein.

MATERIALS AND METHODS

Cells

In the present study, I used eight canine lymphoma and leukemia cell lines derived from dogs with naturally occurring lymphoid malignancies. These included the following B-cell lines: 17-71 (multicentric B-cell lymphoma) [Rosales *et al.*, 1988], CLBL-1 (multicentric B-cell lymphoma) [Rütgen *et al.*, 2010], and GL-1 (B-cell acute lymphoblastic leukemia [ALL]) [Nakaichi *et al.*, 1996]; and T-cell lines: CLC (gastrointestinal T-cell lymphoma) [Umeki *et* al., 2013], CLGL-90 (chronic large granular lymphocytic T-cell leukemia) [Suter *et al.*, 2005], Ema (mediastinal T-cell lymphoma), Nody-1 (alimentary T-cell lymphoma) [Yamazaki *et al.*, 2008], and UL-1 (renal T-cell lymphoma) [Bonnefont-Rebeix *et al.*, 2016]. All canine lymphoma and leukemia cell lines were maintained in a complete medium [RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan)]. I used HEK293T human cell line as a positive control of p16 protein expression for western blot analysis (Fig. 1). HEK293T human cell line was maintained in a complete medium [D-MEM (high glucose) with L-glutamine and phenol red (FUJIFILM Wako Pure Chemical Corporation) containing 10% FBS and 1% penicillin-streptomycin (Nacalai Tesque)]. Live cells equal to or greater than 90% are used to initiate cell culture. Furthermore, I used Mycoplasma-free cells with passage times under thirty. All the cells were maintained at 37 ◦C in humidified air containing 5% CO2.

Treatment of Cell Lines with 5-aza-2-Deoxycytidine

A demethylation drug, 5-aza-20'-deoxycytidine (5-Aza, Sigma–Aldrich, Saint Louis, MO, USA), which causes DNA demethylation, was dissolved in distilled water at a concentration of 43.8 mM and stored at −20 ◦C until required. The treatment of the canine lymphoma cell

lines with 5-Aza according to the indicated doses (CLC and Nody-1 cell lines, 0.25 μM; 17-71, CLBL-1, GL-1, CLGL-90, Ema, and UL-1 cell lines, 0.5 μM) was determined based on our preliminary experiments (Fig. 2). The cells were cultured with or without 5-Aza for 72 h. The medium with or without 5-Aza was changed every 24 h. At the end of the culture period, total RNA and protein amounts were extracted from the cell lines, as described below.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using the NucleoSpin RNA Plus Kit (TakaraBio, Shiga, Japan). Complementary DNA (cDNAs) was synthesized using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Single-strand cDNAs were subjected to real-time PCR amplification using the THUNDERBIRDTM SYBRTM qPCR Mix (TOYOBO) according to the manufacturer's instructions. The quantity of total RNA and cDNA was calculated using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), with the quality assessment of nucleic acid based on the absorbance waveform. The RNA and cDNA were stored in the freezer at −30 ◦C before use. The p16 mRNA expression level was measured by real-time PCR using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with p16 primers (p16F and p16R; amplicon length 95 bp, Table 1) [Fujiwara-Igarashi *et al.*, 2013]. Ribosomal protein L32 (RPL32) was used as an endogenous control (RPL32F and RPL32R; amplicon length 100 bp, Table 1) [Peters *et al.*, 2007]. The cycling protocol was as follows: denaturation step at 95 ◦C for 30 s; 45 cycles of denaturation at 95 ◦C for 5 s, annealing at 60 ◦C for 10 s, and extension at 72 ◦C for 10 s. PCR amplicons were electrophoresed on a 3% agarose gel. The comparative cycle threshold (Ct) method was used to quantify the p16 transcript levels. ΔCt was determined by subtracting the Ct value of RPL32 from that of p16 mRNA. The levels of p16 mRNA relative to RPL32 were calculated as 2−ΔCt. All samples were evaluated in three replicates of the triplicate assay.

Western Blot Analysis

Briefly, canine lymphoma cells were lysed in SDS lysis buffer with protease and phosphatase inhibitors (Thermo Fisher Scientific) and kept on ice or stored at −80 ◦C if not used immediately. Cell lysates were electrophoresed on 6% or 12% SDS polyacrylamide gels at 2 A per gel for one hour. The lysates were then transferred onto a PVDF membrane (Merck Millipore, Billerica, MA, USA) at 100 V for one hour. The PVDF membrane was washed three times in Tris-buffered saline containing 0.1% Tween-20 (TBST), blocked for one hour with 5% nonfat milk/TBST (blocking buffer), and subsequently washed three more times with TBST. The PVDF membrane was then incubated overnight at 4 ◦C with mouse monoclonal anti-p16 (1:500, F-8; Santa Cruz Biotechnology, Dallas, TX, USA) [Murphy *et al*., 2017], rabbit monoclonal anti-phospho-pRb (1:1000, phospho-T826; Abcam, Cambridge, UK), or mouse monoclonal anti-pRb (1:1000, G3-248; BD Pharmingen, San Diego, CA, USA) [Fosmire *et al.*, 2007] diluted in 5% nonfat milk/TBST (antibody dilution). Mouse monoclonal anti-β-actin (1:5000, AC-15; Sigma–Aldrich) diluted in 0.5% non-fat milk/TBST was used as the endogenous control. The antibodies used are summarized in Table 2. The membranes were washed three times for 10 min each and incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody (Santa Cruz Biotechnology) for one hour at room temperature. Finally, membranes were washed three times for 10 min, then incubated for 5 min with SuperSignalTM West Pico PLUS Chemiluminescent Substrate reagent (Thermo Fisher Scientific) and imaged using an AMERSHAM ImageQuant 800 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

Statistical Methods

The real-time PCR values of p16 mRNA expression were assessed using CFX Maestro software (Bio-Rad). p16 protein expression was quantified using ImageJ software [Abramoff *et al.*, 2004]. The p16 mRNA and protein expression were analyzed using nonparametric test

by independent samples Kruskal–Wallis test, for which significance values have been adjusted by Dunn– Bonferroni correction for multiple testing. Furthermore, the p16 mRNA and protein expression values with or without 5-Aza treatment were analyzed using a sample-paired t-test. Statistical significance was set at $P < 0.05$.

RESULTS

Expression of p16 mRNA was Correlated with p16 Protein Expressions

To analyze the expression correlation between the p16 gene and protein, we examined the p16 gene and protein expressions in eight canine lymphoma and leukemia cell lines (B-cells: 17-71, CLBL-1, and GL-1; T-cells: CLC, CLGL-90, Ema, Nody-1, and UL-1) using real-time PCR and western blot analysis, respectively (Fig. 3).

The relative expression levels of p16 mRNA in the CLBL-1, CLC, CLGL-90, Ema, Nody-1, and UL-1 cell lines were between 1.0×10^{-5} and 1.0×10^{-4}. In contrast, those of the 17-71 and GL-1 cell lines were between 1.0×10^{-3} and 1.0×10^{-2} (Fig. 3A), showing that the expression levels of p16 mRNA in the CLBL-1 B-cell line and in the CLC, CLGL-90, Ema, Nody-1, and UL-1 T-cell lines were significantly lower than those in the 17-71 and GL-1 B-cell lines.

Furthermore, Fig. 3B and 3C show the expression levels of the p16 protein observed in the 17-71 and GL-1 B-cell lines, where the p16 mRNA expression levels were significantly high. In contrast, I did not observe p16 protein expression in the CLBL-1 B-cell line or in the CLC, CLGL-90, Ema, Nody-1, and UL-1 T-cell lines, where the expression levels of the p16 gene were low, indicating that the expression levels of the p16 gene and protein are highly correlated.

The 5-Aza Treatment Induced the Expressions of the p16 Gene and Protein; and Decreased pRb Phosphorylation in Some Canine Lymphoma and Leukemia Cell Lines

To determine whether the decreased expression of the p16 gene and protein was associated with the methylation of that gene, the two groups of cell lines were treated with 5-Aza and compared with groups without it using real-time PCR and western blot analysis.

Fig. 4 shows the expression of p16 gene in canine lymphoma cells treated with or without 5-Aza. After 5-Aza treatment, the expression levels of p16 mRNA were significantly increased in B-cells (17-71, CLBL-1, and GL-1) and T-cells (CLC, Nody-1, and UL-1). In contrast, p16 gene expression was not altered in the CLGL-90 and Ema cell lines after 5-Aza treatment.

Fig. 5 and 6 show the expression of the p16 protein, the phosphorylation of pRb, and the total pRb in canine lymphoma cells with or without 5-Aza. In addition to the 17- 71 and GL-1 cell lines, p16 protein expression was observed in the CLBL-1, CLC, Nody-1, and UL-1 cells treated with 5-Aza. In contrast, p16 gene and protein expression levels were not altered and still showed low expression in the CLGL-90 and Ema cell lines after 5-Aza treatment (Figures 2 and 4). The results indicated that p16 protein expression was suppressed by methylation of the p16 gene in the CLBL-1, CLC, Nody-1, and UL-1 cell lines; but not in the CLGL-90 and Ema cell lines

The expression levels of the p16 mRNA in the 17-71 and CLBL-1 cell lines without the 5-Aza treatment, as shown in Fig 3A and 4, were different (for example in 17-71: $8.07 \times 10^{-3} \pm 4.22 \times 10^{-3}$ vs. $6.53 \times 10^{-4} \pm 8.83 \times 10^{-5}$; CLBL-1: $3.64 \times 10^{-5} \pm 2.85 \times 10^{-5}$ vs. $3.77 \times 10^{-4} \pm 1.88 \times 10^{-4}$). In contrast, the expression levels of the p16 protein in the 17-71 and CLBL-1 cell lines without the 5-Aza treatment, as shown in Fig. 3C and 5B, were quite similar (for example in 17-71: 0.49 ± 0.04 vs. 0.87 ± 0.07; CLBL-1: 0 vs. 0).

To examine whether the decreased p16 gene and protein expression was related to the hyperphosphorylation of pRb, I explored the phosphorylation of pRb by western blot analysis using cell lines treated with or without 5-Aza. After 5-Aza treatment, the phosphorylation level of pRb decreased in the CLBL-1 B-cell line, in which p16 protein expression was induced (Fig. 5). In contrast, the pRb phosphorylation was not identified in the 17-71 and GL-1 cells treated

with and without 5-Aza, in which the expression of the p16 protein was high regardless of the 5-Aza treatment.

Fig. 6 shows the phosphorylation of pRb in the T-cell lines. After demethylation treatment, pRb phosphorylation was significantly decreased in the CLC, Nody-1, and UL-1 cell lines, and p16 gene and protein expression were increased (Fig. 6C). In contrast, the CLGL-90 cell line, which had low expression of the p16 protein, still significantly exhibited phosphorylation of pRb after 5-Aza treatment. Moreover, the phosphorylation of pRb was not altered in the Ema cell line.

The total pRb expression was significantly increased in the CLBL-1 B-cell line (Fig. 5D) and the Nody-1 T-cell lines (Fig. 6D) after demethylation treatment using 5-Aza. In contrast, the total pRb expression was not altered in the 17-71, GL-1, CLC, CLGL-90, Ema, and UL-1 cell lines treated with 5-Aza.

DISCUSSION

Inactivation of the p16 gene has been reported in canine lymphoma cells [Fosmire *et al.*, 2007, Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a, Fujiwara-Igarashi *et al.*, 2014b, Modiano *et al.*, 2007]. However, the expression of the p16 protein has not been examined in previous studies. The present study clarified the direct relationship between inactivation of the p16 gene and decreased protein expression; and pRb phosphorylation in canine lymphoma cells. To the best of our knowledge, this is the first report that methylation of the p16 gene results in decreased expression of the p16 protein and pRb phosphorylation in canine tumor cells.

In previous study of canine lymphoma cells, low expression of the p16 gene was reported in the CLBL-1, GL-1, Nody-1, Ema, and UL-1 cell lines, and hypermethylation of the p16 gene was observed in the CLBL-1, GL-1, and UL-1 cell lines [Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a]. Furthermore, in a different report, deletion of the p16 gene and phosphorylation of pRb was confirmed in 100% of canine high-grade T-cell non-Hodgkin lymphoma (NHL) cases [Fosmire *et al.*, 2007].

The p16 gene inactivation by deletions, mutations, and hypermethylation has been reported to be associated with aggressive variants in human NHLs [Pinyol *et al.*, 1998]. In humans, loss of the p16 gene is significantly correlated with the stage of human DLBCL [Baran *et al.*, 2017], *and p16* promoter methylation was detected in 80% of patients with stage III/IV Burkitt's lymphomas [Robaina *et al.*, 2015]. Furthermore, in human DLBCLs, the loss of the p16 (CDKN2A) and TP53 genes was observed in 35% and 8% of patients, respectively, and was significantly associated with shorter survival after rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) treatment [Jardin *et al.*, 2010]. Based on the above, the examination of the inactivation of p16 gene and protein expression, as well as

hyperphosphorylation of Rb proteins, may be further valuable parameters for prognostic decision-making and treatment biomarkers for naturally-occurring canine lymphoma cases.

Table 3 summarizes the p16 gene and protein expression analyses of the p16 gene and protein, and pRb phosphorylation in canine lymphoma cell lines treated with or without 5-Aza. When p16 gene expression was low, the expression of p16 protein was not observed. After 5-Aza treatment, p16 gene and protein expression was increased in the CLBL-1, CLC, Nody-1, and UL-1 cell lines. In addition, the p16 protein expression stably expressing in the 17-71 and GL-1 cells with and without 5-Aza treatment. The phosphorylation of pRb was decreased in the CLBL-1, CLC, Nody-1, and UL-1 cell lines treated with 5-Aza, where the p16 gene and protein expression levels were increased. These results suggest that methylation of the p16 gene and hyperphosphorylation of pRb occurred in the CLBL-1, CLC, Nody-1, and UL-1 cell lines. In contrast, the CLGL-90 and Ema cell lines treated with 5-Aza showed low expression levels of the p16 gene and protein; and phosphorylation of pRb. In contrast, the cell lines in which the p16 gene and protein expression levels were high, 17-71 and GL-1 cells were not related to methylation of the p16 gene and hyperphosphorylation of pRb, suggesting that a mechanism other than inactivation p16 and phosphorylation of pRb is important in these cell lines.

In the present study, I observed low expression of the p16 gene and protein in the CLBL-1, CLC, CLGL-90, Ema, Nody-1, and UL-1 cell lines. I also confirmed higher expression of the p16 gene and protein in the 17-71 and GL-1 cell lines. In contrast, in previous studies, lower expression and hypermethylation of the p16 gene were identified in the GL-1 cell line [Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a]. Although I could not identify the reason for this discrepancy, characteristic alterations may have occurred in the GL-1 cell line during culture at different laboratories.

Deletion of p16 gene and hyperphosphorylation of pRb has been reported to reach 100% in canine high-grade T-cell non-Hodgkin lymphoma (NHL) cases [Fosmire *et al.*, 2007]. Our study showed that phosphorylation of pRb may have occurred in 33.3% of the B-cell lines (CLBL-1; 1/3) and in 100% of the T-cell lines (CLC, CLGL-90, Ema, Nody-1, and UL-1; 5/5). However, 40% of the T-cell lines (CLGL-90 and Ema; 2/5), which had low expression of the p16 gene and protein, were not altered after demethylation. In addition, after 5-Aza treatment, pRb phosphorylation was not altered in these cell lines. I could not determine p16 gene methylation in the CLGL-90 and Ema cell lines; aberrations of the p16 gene, including deletions, may have occurred in these cells. Inactivation of p16 gene due to deletion and methylation has been reported in canine lymphomas [Fosmire *et al.*, 2007, Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a, Fujiwara-Igarashi *et al.*, 2014b]. The deletion status of the p16 gene was not confirmed in our study; and should be determined in the future.

The expression analysis of the p16 gene by the real-time PCR was not stable, but that of the p16 protein by western blot analysis was shown to be steadily expressed. Furthermore, the standard deviations (SDs) of the p16 protein expression levels were smaller than those of the p16 gene expression levels. Moreover, pRb phosphorylation was found to be highly related to p16 protein expression. When p16 protein levels were low, the phosphorylation of pRb was observed. In contrast, when the p16 protein levels were high, pRb phosphorylation decreased. Therefore, our study revealed that it would be better to examine p16 protein expression rather than p16 gene expression to detect the pRb pathway in canine lymphoma cells. These results suggest that the expression of the p16 protein could be a promising biomarker.

Chapter 2

Decreased sensitivity of cyclin-dependent kinase 4/6 inhibitors, palbociclib and abemaciclib to canine lymphoma cells with high p16 protein expression and low retinoblastoma protein phosphorylation

SUMMARY

Canine lymphoma/leukemia cell lines with p16 protein expressions: high (17-71 and GL-1) and low (CLBL-1, CLC, Nody-1, and UL-1) were treated *in vitro* with cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors, palbociclib or abemaciclib. Cell proliferation decreased as a result, with higher IC_{50} levels observed in the high p16 (17-71 and GL-1) and one low p16 (UL-1) cell lines compared with the low p16 cells (CLBL-1, CLC, and Nody-1). As expected, palbociclib and abemaciclib treatment reduced pRb phosphorylation in a dose-dependent manner, especially in cells with low p16. These results suggest that CDK4/6 inhibitors have potential as new chemotherapeutic agents for canine lymphoma and high p16 protein expression may be used as a biomarker for resistance to CDK4/6 inhibitor therapy.

INTRODUCTION

The endogenous cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitor, p16 (also called CDKN2A), inhibits cyclin-CDK4/6 [Canepa *et al.*, 2007], resulting in G1-S cell cycle arrest by inhibiting the phosphorylation of retinoblastoma protein (pRb) [Green *et al.*, 2019]. Dysregulation of the p16-pRb pathway results in increased cell proliferation [Hamilton *et al*., 2016] and has been implicated as the impetus of many cancers [Belinsky *et al.*, 2006; Chen *et al.*, 2015, Cui *et al.*, 2015, Luo *et al.*, 2006].

CDK4/6 inhibitors (CDK4/6i), namely palbociclib and abemaciclib, are novel effective therapies approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of human breast cancers in combination with other therapeutics [O'Leary *et al*., 2016, Parylo *et al.*, 2019]. Their inhibitive properties affect cancer cell proliferation, blocks the progression from G1 to S phase, and may inhibit the metastatic potential of cancer cells [Kollmann *et al.*, 2019]. In human cancer cell lines, it has been reported that CDK4/6i can effectively inhibit the proliferation of tumor cells that lose endogenous inhibition of CDK4/6 due to p16 inactivation [Konecny *et al.*, 2011, Li *et al*., 2019]. In canine studies, CDK4/6i and palbociclib have antitumor effects on canine mammary tumor cells [Schoos *et al.*, 2019] and could potentially be used as new anti-cancer treatments for canine melanoma [Bongiovani *et al.*, 2021]; however, there is no data available simultaneously analyzing the expression of p16 protein and phosphorylated pRb (pRb-P).

Decreased expression of p16 has been shown to be common in established canine lymphoma cell lines and lymphoma cells obtained from naturally occurring clinical cases [Fosmire *et al.*, 2007, Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a, Fujiwara-Igarashi *et al.*, 2014b]. In chapter 1, I have found that certain canine lymphoma cell lines simultaneously exhibit p16 gene methylation, loss of p16 protein expression, and pRb

hyperphosphorylation [Maylina *et al.*, 2022], suggesting that the p16-pRb pathway is one of the most crucial mechanisms in canine lymphomagenesis. Moreover, I also found that the expression level of the p16 protein was more strongly correlated with the pRb-phosphorylation level than with p16 mRNA. Therefore, the current study focused on CDK4/6i (palbociclib and abemaciclib) for the treatment of canine lymphoma cells as potential new drugs and explored the correlation between drug sensitivity and expression levels of the p16 protein and pRb-P in canine lymphoma cell lines.

MATERIALS AND METHODS

Cells

Six canine lymphoma/leukemia cell lines were used based on our previous study [Maylina *et al.*, 2022]: two cell lines showed high p16 protein expression (17-71 [Rosales *et al.*, 1988] and GL-1 [Nakaichi *et al.*, 1996]) and four cell lines showed low p16 protein expression (CLBL-1 [Rütgen *et al.*, 2010], CLC [Umeki *et al.*, 2013], Nody-1 [Yamazaki *et al.*, 2008], and UL-1 [Yamazaki *et al.*, 2008]). All canine lymphoma/leukemia cell lines were maintained in complete medium (RPMI-1640; FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) maintained at 37 °C in humidified air containing 5% CO_2.

Cell Counting Kit-8 (CCK-8)

The half-maximal inhibitory concentration (IC_{50}) of each drug using the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Each cell line was treated with palbociclib (Sigma-Aldrich, Saint Louis, MO, USA) or abemaciclib (LKT Laboratories Inc., Saint Paul, MN, USA) at various concentrations (0, 0.01, 0.1, 1, 10, and 100 μM) in 96-well plates (3 wells/group) for 48 hr. Briefly, cell proliferation was assessed by adding 10 μL WST-8(2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) and incubated for 4 hr. Absorbance was measured at a wavelength of 450 nm. The IC_{50} of each drug was calculated from drug survival curves.

Western Blot Analysis

Cell lysates were electrophoresed on 6% or 12% SDS polyacrylamide gels at 2 A per gel for one hr. The electrophoresed proteins were transferred onto a polyvinylidene fluoride

(PVDF) membrane (Merck Millipore, Billerica, MA, USA) at 100 V for one hr. The PVDF membrane was washed in Tris-buffered saline containing 0.1% Tween-20 (TBST), blocked for one hr with 5% non-fat milk/TBST (blocking buffer), and washed with TBST. The PVDF membrane was incubated overnight at 4°C with mouse monoclonal anti-p16 (1:500, F-8; Santa Cruz Biotechnology, California, USA) [Murphy *et al.*, 2017] and rabbit monoclonal anti-phospho-pRb (1:1000, phospho-T826; Abcam, Cambridge, UK) diluted in 5% non-fat milk/TBST (antibody dilution) [Fosmire *et al.*, 2007]. A mouse monoclonal anti-β-actin antibody (1:5000 dilution of 0.5% non-fat milk/TBST, AC-15; Sigma-Aldrich) was used as the endogenous control. The PVDF membranes were washed thrice for 10 min each and incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody (Santa Cruz Biotechnology) at room temperature for one hr. The membranes were then washed thrice for 10 min. Before visualization, the membranes were incubated for 5 min using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate reagent (Thermo Fisher Scientific). Proteins on the membranes were captured using an AMERSHAM ImageQuant 800 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Statistical Methods

The IC_{50} values of palbociclib and abemaciclib were analyzed using one-way ANOVA and Tukey's *post-hoc* test. The p16 protein expression was quantified using ImageJ software [Abramoff *et al.*, 2004]. The quantification data of ImageJ are expressed as the mean and standard deviation (SD) values and were then analyzed by one-way ANOVA and Tukey's *post-hoc* test. $P < 0.05$ was set as significant statistical values.

RESULTS

High Expression of p16 Protein was Correlated to Low Sensitivity of CDK4/6 Inhibitors Palbociclib and Abemaciclib Treatment

To explore the relationship between p16 protein expression and sensitivity to palbociclib and abemaciclib, I first examined the IC_{50} of each drug using the cell counting kit-8 (CCK-8) assay. The proliferation of palbociclib- and abemaciclib-treated cells was lower in the cell lines with low p16 (CLBL-1, CLC, and Nody-1) than in those with high p16 (17-71 and GL-1) and one low p16 (UL-1) [Fig. 7A]. In cell lines with high p16 (17-71 and GL-1), the IC_{50} values of abemaciclib were 1.6378 ± 0.1374 μM and 0.4060 ± 0.0957 μM, respectively, and those of abemaciclib were 0.1316 ± 0.0142 μM and 0.0330 ± 0.0082 μM, respectively (Table 4, Fig. 7B, C). In cell lines with low p16 (CLBL-1, CLC, Nody-1, and UL-1), the IC_{50} values of palbociclib were 0.0081 ± 0.0013, 0.0061 ± 0.0004, 0.0086 ± 0.0013, and 1.1742 ± 0.0844 (μM), respectively, and those of abemaciclib were 0.0053 ± 0.0005, 0.0068 ± 0.0003, 0.0089 ± 0.009, and 0.1128 ± 0.0018 (μM), respectively. In summary, the IC_{50} values of palbociclib and abemaciclib in cells with low p16 (CLBL-1, CLC, and Nody-1), except UL-1, were consistently lower than those in cells with high p16 (17-71 and GL-1), suggesting that cells with high p16 were relatively resistant to CDK4/6i. This is concordant with a previous human study reporting that IC_{50} values of the p16 unmethylated (p16 expressing) cell lines were consistently higher than those of the p16 methylated (p16 low-expressing) cell lines and p16 deleted cell lines (18.88 vs. 4.61 and 3.99 [μM]), respectively] in human lung and gastric cancer cell lines treated with palbociclib [Li *et al.*, 2019].

CDK4/6 Inhibitors Palbociclib and Abemaciclib Treatment Reduced pRb Phosphorylation in a Dose-dependent Manner in Low p16 Canine Lymphoma Cells

As shown in chapter 1 [Maylina *et al.*, 2022], the p16 protein expression was observed in 17-71 and GL-1 without CDK4/6i, but not in the other cell lines (CLBL-1, CLC, Nody-1, and UL-1, Fig. 8A–F). The pRb-P was barely identified in 17-71 and GL-1 without CDK4/6i, but was clearly found in the other cell lines. Phosphorylation of pRb was reduced significantly with 0.01 μM palbociclib or abemaciclib for 12 h, and it was reduced as the concentration was increased except in 17-71 with no expression of pRb-P with or without CDK4/6i. My study revealed that palbociclib and abemaciclib treatment reduced pRb phosphorylation in a dose-dependent manner in canine lymphoma cells, especially in cells with low p16 and high pRb-P (CLBL-1, CLC, Nody-1, and UL-1), suggesting that CDK4/6i inhibits the phosphorylation of pRb by inhibiting CDK4/6.

DISCUSSION

I explored IC_{50} values of CDK4/6i, palbociclib and abemaciclib to six canine lymphoma/leukemia cell lines (high p16 cells: 17-71 and GL-1; low p16 cells: CLBL-1, CLC, Nody-1 and UL-1). Although there were no reports using canine lymphoma/leukemia cells, the IC_{50} values of 64.06 nM for P114 cells and 18.8 nM for CF41 cells has been reported in canine mammary tumors treated with palbociclib, respectively [Schoos *et al.*, 2019]. Another report found that cell proliferation decreased significantly on canine melanoma cells after being treated with palbociclib (1 μM for LMCK and OLGA cells and 2.5 μM for CMM10 cell). However, in CMM12 canine melanoma cells, cell proliferation remained relatively constant with increasing concentrations of CDK4/6i, and a significant reduction in cell proliferation was observed only at the maximum palbociclib concentration tested (10 μM) [Bongiovani *et al.*, 2021]. Compared with these cells, the canine lymphoma cells showed higher sensitivity to palbociclib, particularly in three of the four cell lines with low p16, as their IC_{50} values were less than 10 nM.

I found that high p16-represented cells (17-71 and GL-1) showed low sensitivity to palbociclib and abemaciclib. A study has reported the biological sensitivity of engineered isogenic cells, indicating that high levels of p16 predict insensitivity to palbociclib [Green *et al.*, 2019]. In contrast, I found that one of the low p16 cell lines, UL-1, showed a higher IC_{50} similar to that of high p16 cells. I speculated that UL-1 cells may be intrinsically resistant to CDK4/6i owing to pRb deficiency or downstream bypass [Green *et al.*, 2019, Knudsen *et al.*, 2017, Witkiewicz *et al.*, 2011]. However, as a limitation of this study, I need to further explore the mechanism(s) that mediate CDK4/6i resistance in UL-1 cells.

In conclusion, CDK4/6 inhibitors exhibit potential as new chemotherapeutic agents for canine lymphoma and high p16 protein expression may be used as a useful biomarker for resistance to CDK4/6 inhibitor therapy. Further studies are necessary to verify CDK4/6

inhibitors including palbociclib and abemaciclib as the potential new chemotherapeutic agents using a transplantation mouse model of canine lymphoma *in vivo*

CONCLUSION

In canine lymphoma, inactivation of the endogenous CDK4/6 inhibitor, p16, was correlated with the phosphorylated Rb protein [Fosmire *et al.*, 2007], resulting in increased cell proliferation [Ohtani *et al.*, 2004] and poor prognosis [Modiano *et al.*, 2007]. It was suggested that the p16-pRb pathway might be a valuable parameter for canine lymphoma. In human, palbociclib and abemaciclib are CDK4/6 inhibitor drugs that have been reported for cancer treatment that effectively reduces the phosphorylated pRb and inhibits the proliferation of cancer cells with p16 inactivation [Chen *et al.*, 2012, Konecny *et al.*, 2011, Li *et al.*, 2019, Wiedemeyer *et al.*, 2010, Young *et al.*, 2014]. Therefore, the establishment of a new treatment strategy using CDK4/6 inhibitors is very important for canine lymphoma patients in the future.

In chapter 1 of this thesis, I verified the correlation between the inactivation of the p16 gene and its protein expression with the methylation of that gene, as well as hyperphosphorylation of the Rb protein. I revealed that the pRb phosphorylation was found to be highly related to the p16 protein expression, since p16 protein was shown to be steadily expressed and the standard deviations of the p16 protein expression levels were smaller than those of the p16 gene expression levels. When the p16 gene expression was high, the p16 protein was exhibited, and the phosphorylated pRb was barely identified (Fig. 9A), in contrast, when the p16 gene expression was low, the expression of the p16 protein was not observed, and the phosphorylated pRb was clearly detected (Fig. 9B). I observed low expression of the p16 gene and protein in the CLBL-1, CLC, CLGL-90, Ema, Nody-1, and UL-1 cell lines, and confirmed higher expression of the p16 gene and protein in the 17-71 and GL-1 cell lines. Treatment with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza) revealed that the expression levels of the p16 gene and protein were significantly increased and phosphorylated pRb was decreased (Fig. 10) in CLBL-1, CLC, Nody-1, and UL-1, suggesting that the methylation of the p16 gene results in decreased expression of the p16 protein and hyperphosphorylated Rb protein in these canine lymphoma cells. Based on the chapter 1

results, the examination of the p16 protein expression, as well as the hyperphosphorylation of Rb proteins, may be further valuable parameters for the p16-pRb pathway and treatment biomarkers related to CDK4/6 inhibitors in canine lymphoma.

In chapter 2 of this thesis, I explored the correlation between CDK4/6 inhibitors (palbociclib and abemaciclib) sensitivity with various expression levels of the p16 protein and phosphorylated pRb using the canine lymphoma cell lines. I found that the IC_{50} values of palbociclib and abemaciclib in cells with low p16 (CLBL-1, CLC, and Nody-1), except UL-1, were consistently lower (less than 10 nM) than those in cells with high p16 (17-71 and GL-1), suggesting that low p16 cells were sensitive (Fig. 11A) and high p16 cells were relatively resistant (Fig. 11B) to CDK4/6 inhibitors. My study revealed that palbociclib and abemaciclib treatment reduced pRb phosphorylation in a dose-dependent manner in canine lymphoma cells, especially in cells with low p16 (CLBL-1, CLC, Nody-1, and UL-1). Based on the chapter 2 results, CDK4/6 inhibitors show potential as new chemotherapeutic agents for canine lymphoma and high p16 protein expression may be used as a useful biomarker for treatment resistance of CDK4/6 inhibitors.

In my study, I found the p16 expression was high in the GL-1 cell line and pRb-P was barely identified, suggesting that the cell was not related to the methylation of the p16 gene. However, lower expression and hypermethylation of the p16 gene were reported in the GL-1 cell line [Fujiwara-Igarashi *et al.*, 2013, Fujiwara-Igarashi *et al.*, 2014a]. I could not identify the reason for this discrepancy, however, I speculated that during culture at different laboratories, characteristic alterations may have occurred in the GL-1 cell line. My study showed the p16 gene and protein, as well as phosphorylated Rb protein expression, was not altered in the CLGL-90 and Ema cell lines after the demethylating treatment with 5-Aza, suggesting that another p16 inactivation mechanism and phosphorylation of pRb are important in these cell lines. I found one of the low p16 cell lines, UL-1, showed a higher IC_{50} similar to

those of high p16 cells, suggesting that UL-1 cells may be intrinsically resistant to CDK4/6 inhibitors in other mechanisms. Although some cell lines showed exceptions, in this thesis, I revealed the p16 protein expression is well correlated with pRb phosphorylation in canine lymphoma cells. Furthermore, I represented that the CDK4/6 inhibitors exhibited as a new potential chemotherapeutic agent in canine lymphoma, as well as high p16 protein expression, may be used as a useful biomarker for the treatment resistance of CDK4/6 inhibitors. Although further studies to explore the mechanism(s) related to the p16 expression and the CDK4/6 inhibitor resistance were necessary, with the best of my knowledge, these results provide important information on the valuable simultaneous examination of p16 and hyperphosphorylated Rb protein expression as a treatment biomarker for canine lymphoma patients in the future.

AKCNOWLEDGMENTS

This doctoral thesis would not have been possible without the support of countless people.

First and foremost, words cannot express my sincere gratitude and deepest appreciation to Professor Masaru Okuda, my excellent supervisor, esteemed advisor, and chair of my committee for his invaluable patience, guidance, support, and feedback. He is the true definition of an ideal teacher, a leader, and the ultimate role model, offering advice and encouragement with a perfect blend of insight and his unassuming approach to research and science is a source of inspiration, which is something I hope to carry forward throughout my career. I am extremely grateful that he took me on as a student and continued to have faith in me over the years.

I extremely appreciate my co-supervisor, Dr. Kenji Baba for his great feedback, excellent encouragement, noble guidance, tons of advice, and motivating enthusiasm. He arranged extraordinary experiences for me and for providing opportunities for me to grow professionally. I am greatly thankful to my co-supervisor, Professor Yasuyuki Endo, for his continuous support.

I am glad of and grateful for, my time working with Dr. Satoshi Kambayashi, whose guidance from the initial step of research enabled me to develop an understanding of the subject. It is an honor to learn from his brilliant, skillful guidance, stimulation inspiration, and excellent cooperation, which enriched my research higher than my expectation.

I gratefully recognize the help of Professor Takuya Mizuno, Professor Masahiro Morimoto, and Dr. Masaya Igase. Their encouraging words and thoughts, tons of suggestions, and detailed feedback have been very important to me.

I acknowledge the generous research financial support in part by a Grant-in-Aid for Scientific Research (Grant Number: 18K05972) from the Japan Society for the Promotion of Science.

The generous support from the JASSO scholarship (2019-2020), Yamaguchi University scholarship (2020-2021), and Rotary Yoneyama scholarship (2021-2023), which financed and supported me during my doctoral course. My sincere gratitude to my best counselor Ms. Takae Akase, as well as the President and Secretary of Yamaguchi South Rotary Club [Mr. Yasushi Ogaswara and Mr. Akinori Arayama (2020-2021), Ms. Sachie Irie and Mr. Hiroyashu Omi (2021-2022), Ms. Keiko Uchida and Mr. Toru Takeshita (2022-2023)], which I am fortunate to have been a part of the Rotary Club family during past two years.

I also could not have undertaken my journey without a lot of assistance from Joint Graduate School of Veterinary Medicine and International Student Support Office, Yamaguchi University.

I am grateful to all Laboratory of Internal Medicine members, and also, the Japanese families (especially Mrs. Saito's family), as well as, Yamaguchi International Exchange Association (YIEA) and local Japanese communities (especially Kazenokai) who always give me the bigest trust and the greatest support for many positive activities, in addition, numerous friends who endured this long process with me, always offering support and love.

Last, but not least, my warm and heartfelt thanks go to my family for the tremendous support and hope they had given to me. This achievement dedicates to my father (RIP), in addition, especially to my mother (RIP), who passed away during my study in Yamaguchi University, while they were still alive, they never hesitated to express their best wishes and prayers for me. Thank you to my spouse, for his endless support, and for always standing behind me with no exception. Thank you to my son and daughter, for their unconditional and unequivocal love, and are always supportive of my adventures. My deepest thanks to my parent-in-law, my sister, and all my family, who keep me grounded and remind me what is important loving support in our family. Thank you all for the strength they gave to me.

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TABLES

Table 1. Primer sequences used for canine p16 and RPL32 genes.

Target Gene	Primer Name	Sequence (5'-3')	Product Length (bp)	GeneBank Accession No.
p16	16F	GGTCGGAGCCCGATTCA	95	AB675384
	16R	ACGGGGTCGGCACAGTT		
RPL32	RPL32F	TGGTTACAGGAGCAACAAGAA	100	XM848016
	RPL32R	GCACATCAGCAGCACTTCA		

Table 2. Antibodies used for the detection of canine p16, phospho-pRb, pRb and β-actin proteins.

Target Protein	Clone	Manufacture	Diluted with	Dilution
p16	F-8	Santa Cruz Biotechnology	5% nonfat milk/TBST	1:500
phospho-pRb	Phospho-T826	Abcam	5% nonfat milk/TBST	1:1000
pRb	G3-248	BD Pharmingen	5% nonfat milk/TBST	1:1000
β-actin	AC-15	Sigma–Aldrich	0.5% nonfat milk/TBST	1:5000

Table 3. Summary of the expression analysis of the p16 gene and protein, and the pRb phosphorylation in canine lymphoma cell lines treated with (+) or without 5-Aza (−).

Canine Lymphoma Cell Lines		p16					Phospho-pRb		
		Gene Expression		Protein Expression		Methylation	Protein Expression		Hyper-Phosphorylation
		5-Aza					5-Aza		
		(−)	(+)	(−)	(+)		(−)	(+)	
B-cell lines	17-71	++	+++	++	++	−	−	−	−
	CLBL-1 *	++	+++	−	+	+	++++	++	+
	GL-1	+++	++++	+++	+++	−	−	-	−
T-cell lines	CLC *	+	++++	−	+++	+	++	+	+
	CLGL-90	+	+	−	−	−	++	+++	+
	Ema	+	+	−	−	−	++	++	+
	Nody-1 *	+	+++	−	+++	+	++	+	+
	UL-1 *	+	+++	−	+++	+	++	+	+

Tabel 4. Palbociclib and Abemaciclib IC_{50} (µM) values in canine lymphoma cells with high or low p16 expressions.

p16 expression	Cell lines	Palbociclib IC_{50} (µM)	Abemaciclib IC_{50} (µM)
High p16	17-71	1.6378 ± 0.1374	0.1316 ± 0.0142
	GL-1	0.4060 ± 0.0957	0.0330 ± 0.0082
Low p16	CLBL-1	0.0081 ± 0.0013	0.0053 ± 0.0005
	CLC	0.0061 ± 0.0004	0.0068 ± 0.0003
	Nody-1	0.0086 ± 0.0013	0.0089 ± 0.009
	UL-1	1.1742 ± 0.0844	0.1128 ± 0.0018

*) The proliferation and cytotoxicity assay were assessed using CCK8 assay.

*) The data are expressed as the mean and standard deviation (SD) values of three replicates in the triplicate assay. *$P < 0.05$; **$P < 0.01$.

FIGURES

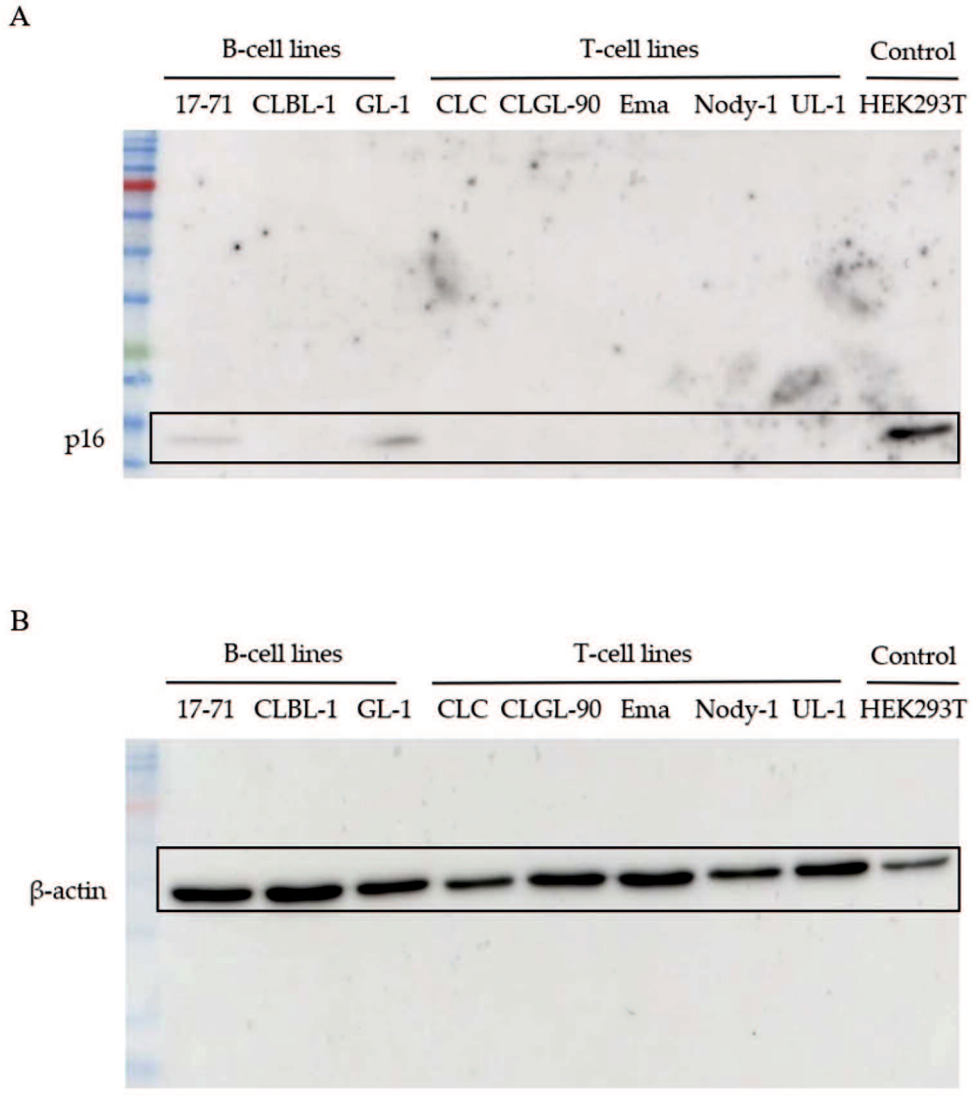


Fig. 1. The expression of the p16 protein in canine lymphoma and leukemia cell lines.
The protein expression was assessed using the western blot analysis. Human HEK293T cell line was used as a positive control of p16 protein expression (A); β-actin was used as the endogenous control (B).

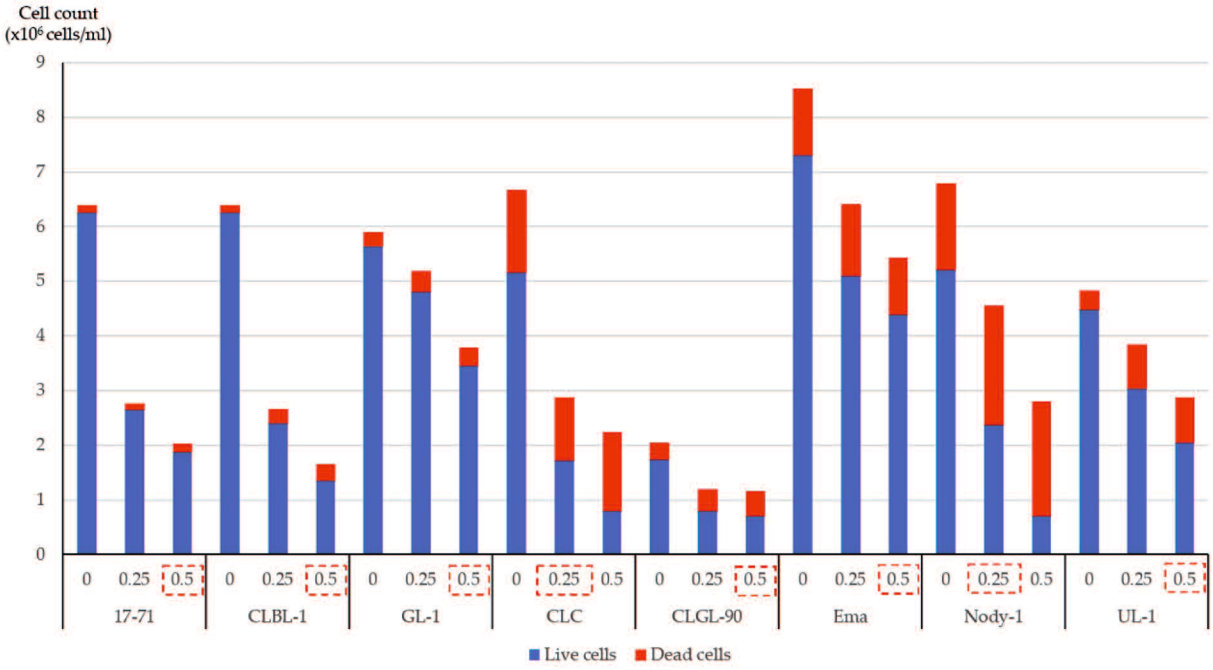


Fig. 2. Cell viability of canine lymphoma and leukemia cell lines after treated with 5-Aza (0.25 µM and 0.5 µM) and without it (0 µM).

After treated with 5-Aza for 72h, total live cells equal to or more than 50% was considered for western blots analysis and real-time PCR [Fujiwara-Igarashi *et al*., 2014a].

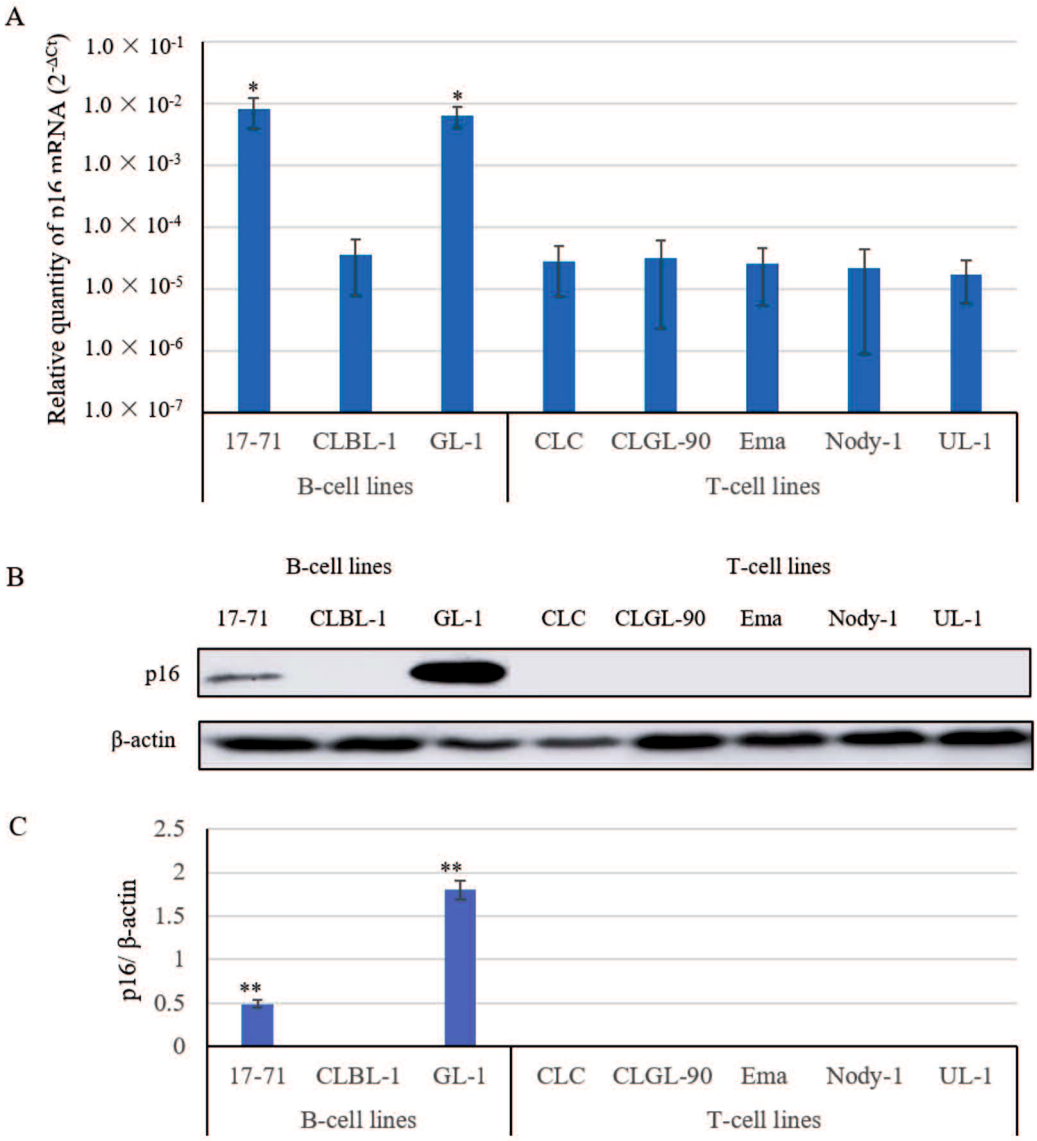


Fig. 3. The expression analysis of the p16 gene and protein in canine lymphoma B- and T-cell lines.

A. The relative expression levels of p16 mRNA were assessed using real-time PCR. The data are expressed as the mean and standard deviation (SD) values of three replicates in the triplicate assay. * $P < 0.05$. B: The expression analysis of the p16 protein in canine lymphoma cell lines. The protein expression was assessed using western blot analysis [B] and quantified using ImageJ [C]. ** $P < 0.01$.

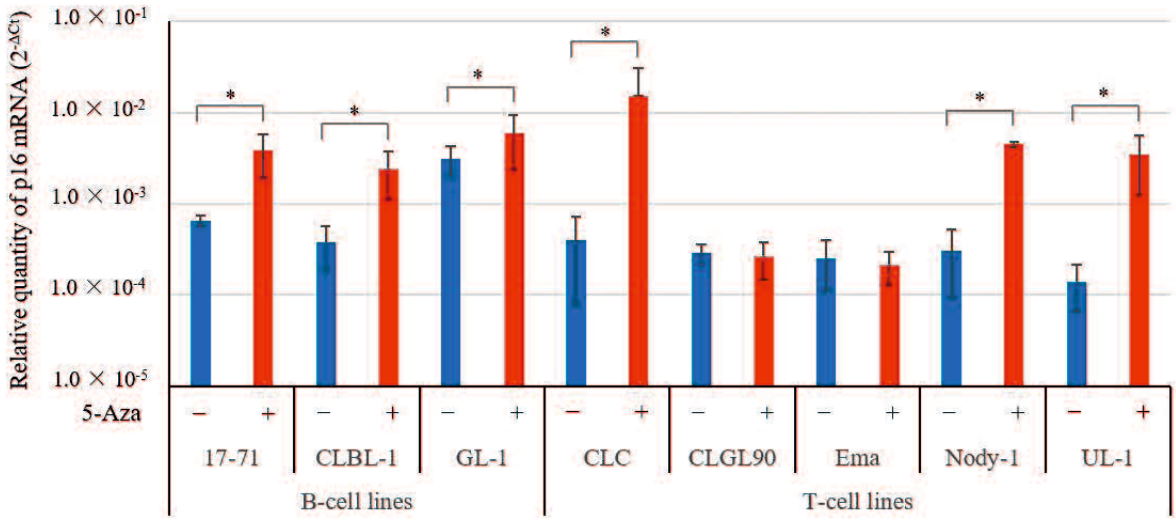


Fig. 4. The expression of the p16 gene in canine lymphoma and leukemia B- and T-cell lines treated with 5-Aza.

The relative expression levels of p16 mRNA in the 17-71, CLBL-1, and GL-1 B-cell lines; and the CLC, Nody-1, and UL-1 T-cell lines were significantly increased after the 5-Aza treatment, as assessed using real-time PCR. The data are expressed as the mean and SDs values of three replicates in the triplicate assay. * $P < 0.01$.

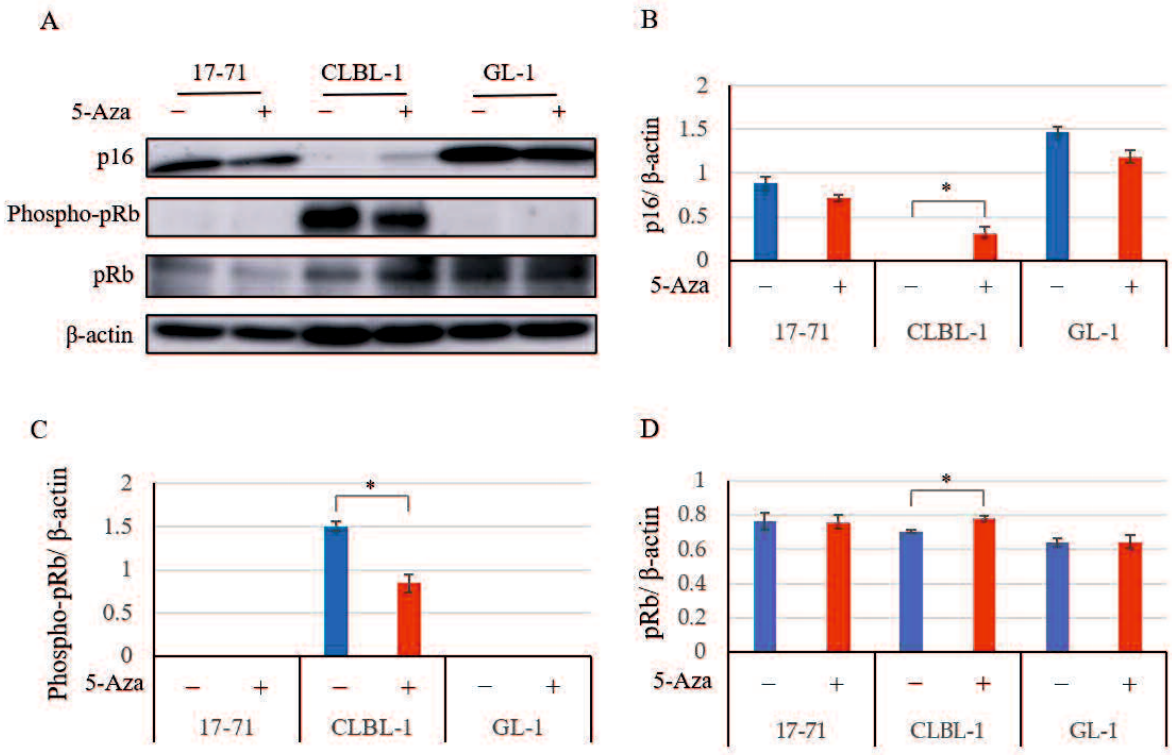


Fig. 5. The expression of the p16 protein in canine lymphoma and leukemia B-cell lines treated with or without 5-Aza.

The protein expression was assessed using the western blot analysis (a) and quantified using ImageJ for p16 (b), phospho-pRb (c), and total pRb (d). β-actin was used as the endogenous control. The data are expressed as the mean and SD values. * $P < 0.01$.

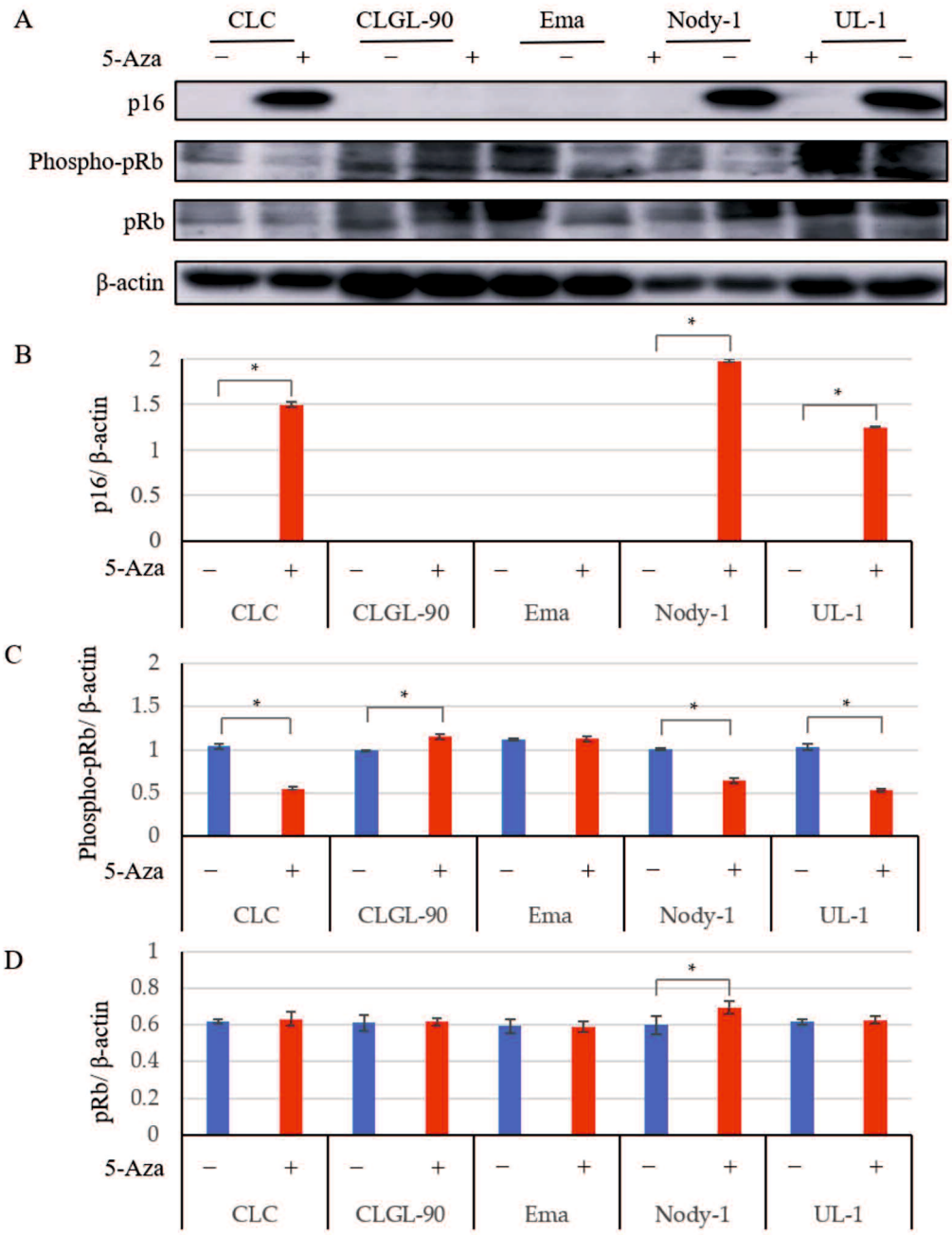
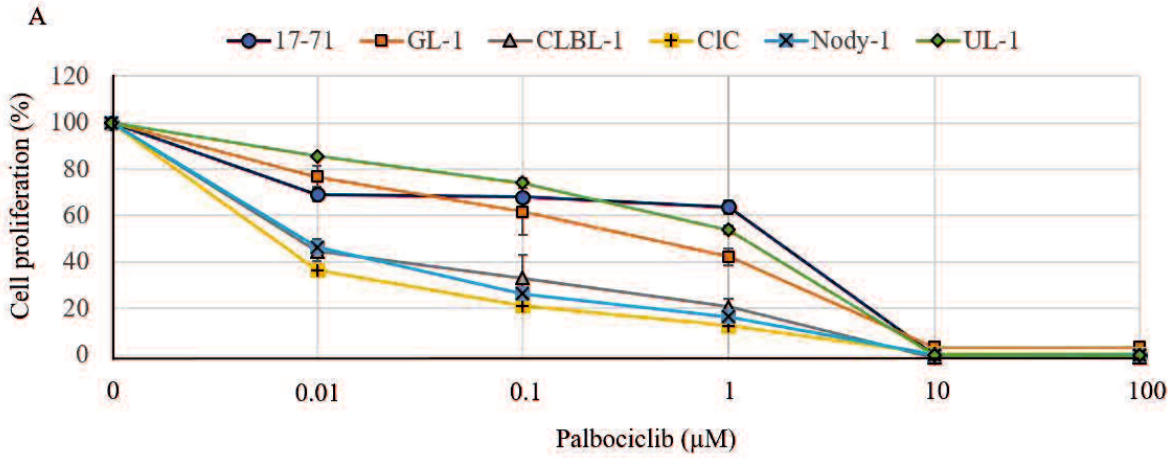
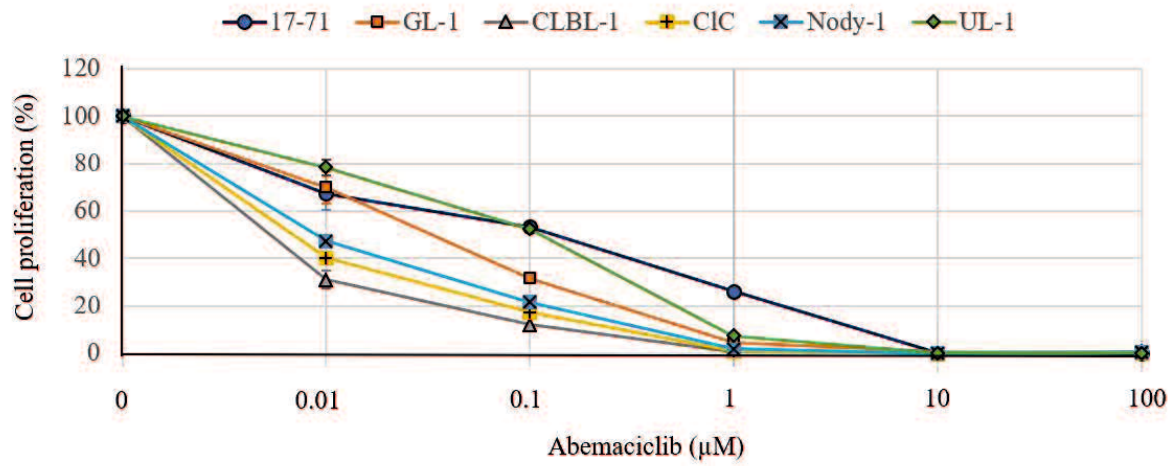


Fig. 6. The expression analysis of the p16 protein in canine lymphoma T-cell lines treated with or without 5-Aza.

The protein expression was assessed using the western blot analysis (a) and quantified using ImageJ for p16 (b), phospho-pRb (c), and total pRb (d). β-actin was used as the endogenous control. The data are expressed as the mean and SD values. * $P < 0.01$.

A


17-71
GL-1
CLBL-1
ClC
Nody-1
UL-1
Cell proliferation (%)
120
100
80
60
40
20
0
0
0.01
0.1
1
10
100
Palbociclib (μM)


17-71
GL-1
CLBL-1
ClC
Nody-1
UL-1
Cell proliferation (%)
120
100
80
60
40
20
0
0
0.01
0.1
1
10
100
Abemaciclib (μM)

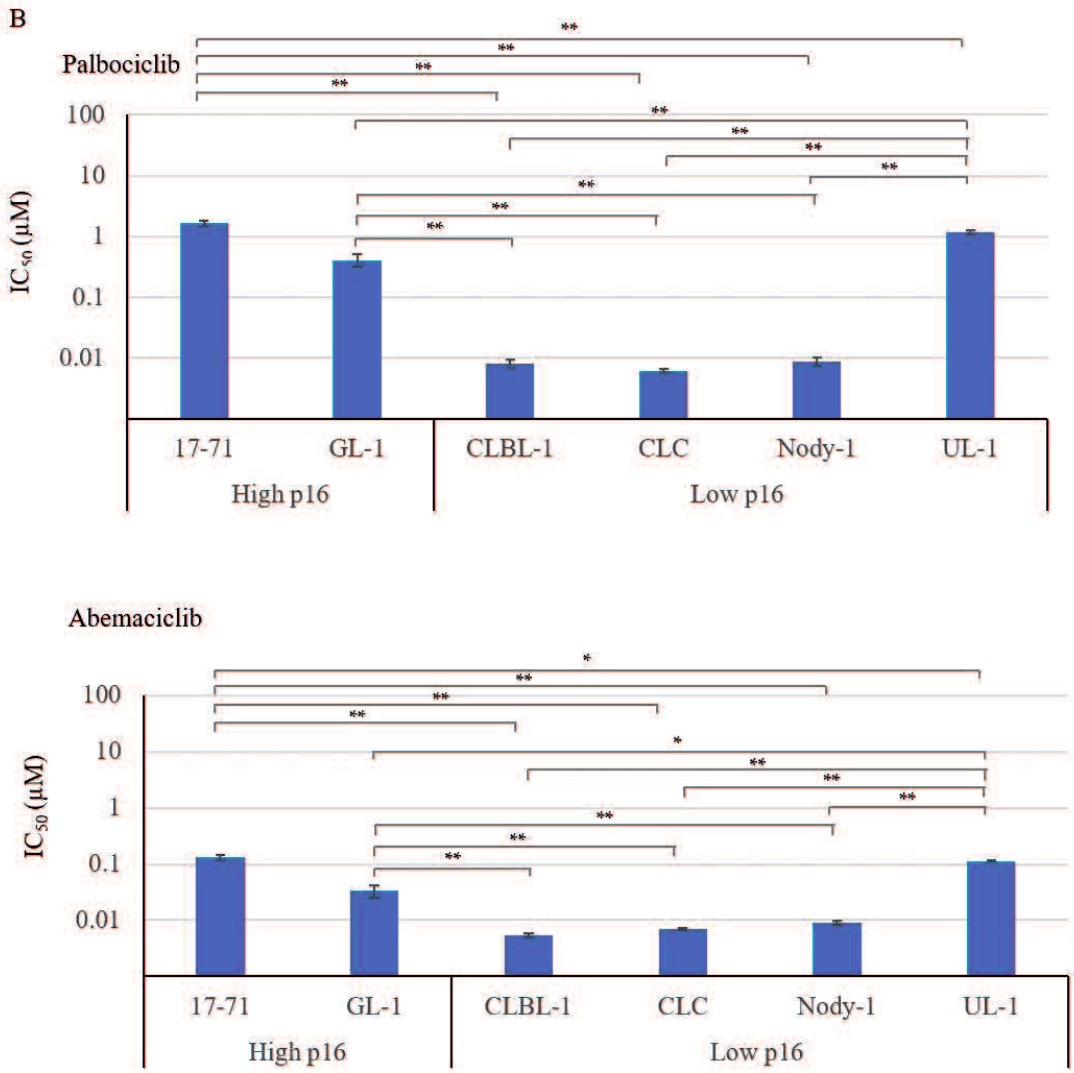
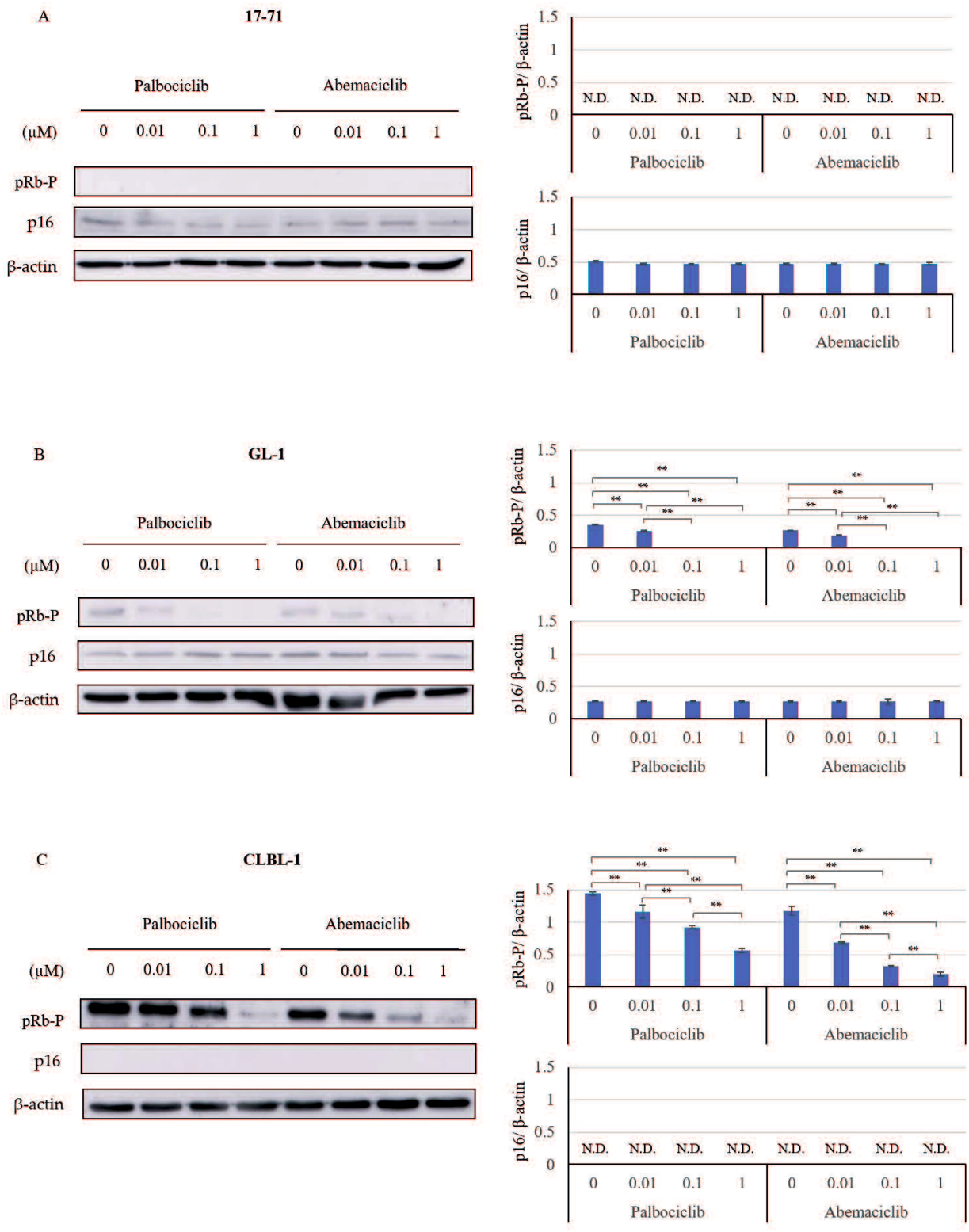


Fig. 7. Association between p16 expression and the sensitivity of CDK4/6 inhibitors palbociclib and abemaciclib in canine lymphoma cells.

(A) Cell proliferation (%) curve in canine lymphoma cells treated with palbociclib (upper) or abemaciclib (lower) at various concentrations. (B, C) Palbociclib and Abemaciclib IC_{50} (μM) values in canine lymphoma cells with high or low p16 expressions. The proliferation and cytotoxicity assay were assessed using CCK8 assay. The data are expressed as the mean and standard deviation (SD) values of three replicates in the triplicate assay. *$P < 0.05$; **$P < 0.01$.


A
17-71
Palbociclib
Abemaciclib
(μM)
0 0.01 0.1 1 0 0.01 0.1 1
pRb-P
p16
β-actin
pRb-P/ β-actin
p16/ β-actin
N.D.
B
GL-1
C
CLBL-1

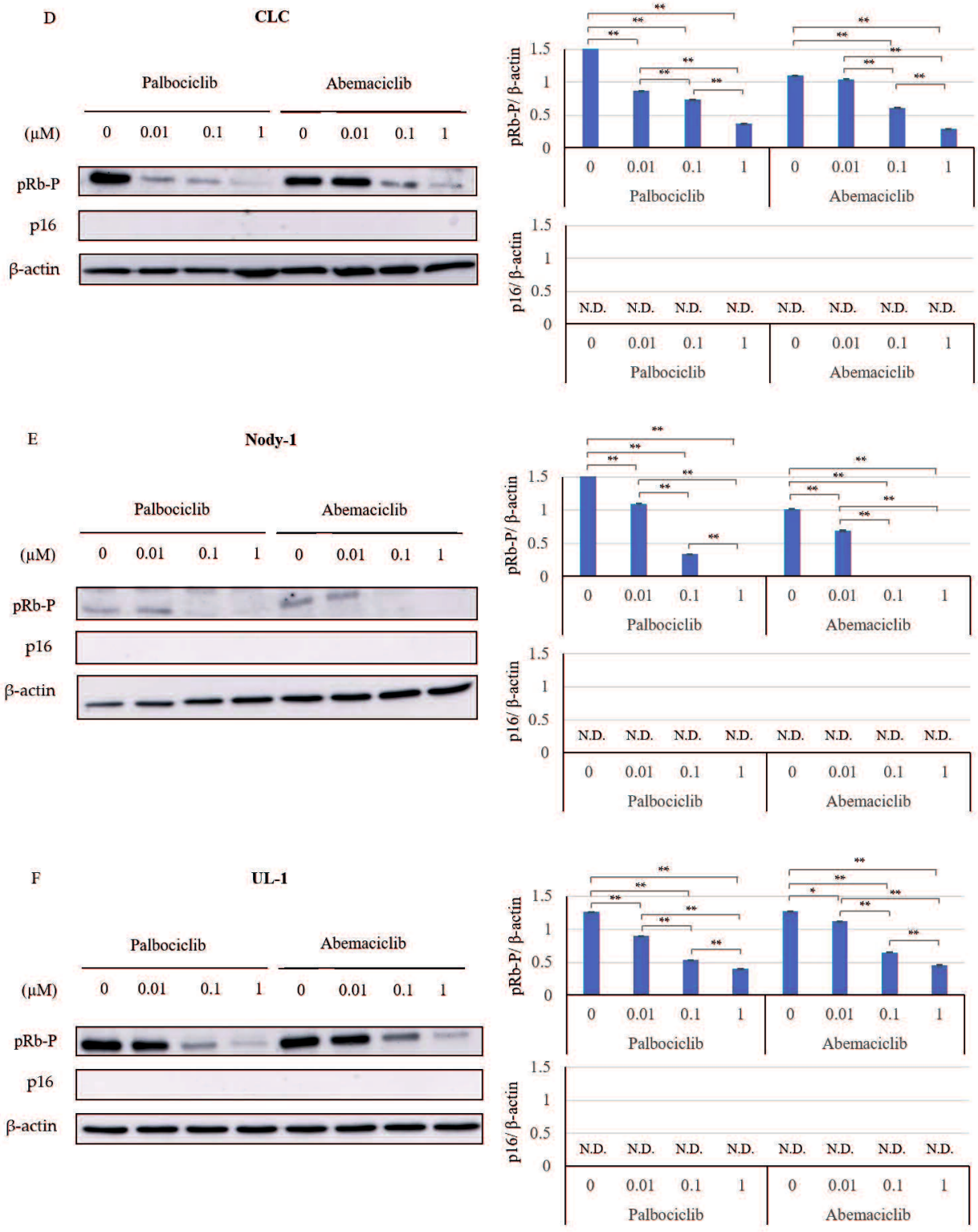


Fig. 8. Palbociclib and abemaciclib inhibit pRb phosphorylation in a dose-dependent manner. Association of palbociclib and abemaciclib treatment to the pRb phosphorylation in canine lymphoma cells with high p16 (A) 17-71 and (B) GL-1; and low p16 (C) CLBL-1, (D) CLC, (E) Nody-1, and (F) UL-1. Phosphorylated pRb (pRb-P) and p16 protein of canine lymphoma cells were assessed using western blot analysis and quantified using ImageJ software. *P < 0.05; **P < 0.01. N.D.; Not detected.

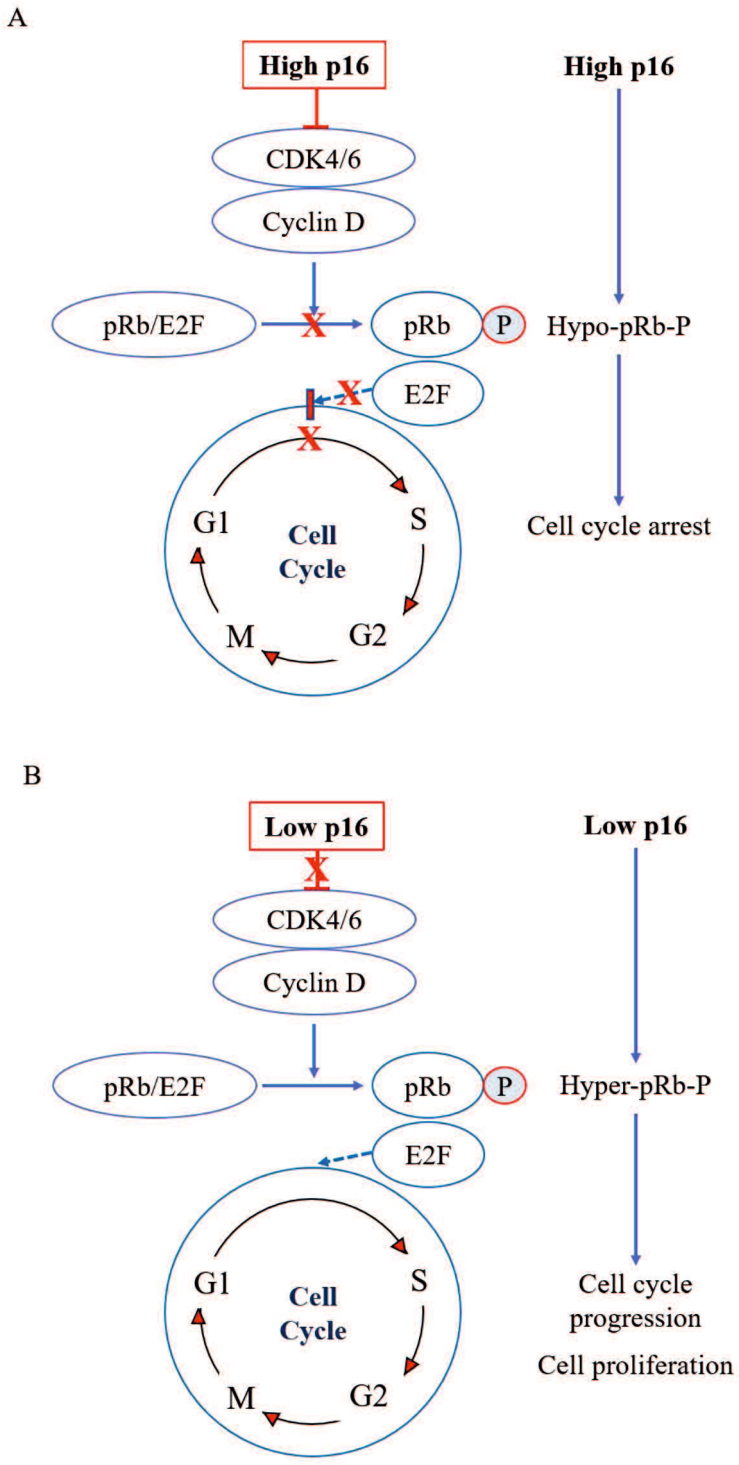


Fig. 9. The relationship between the low or high p16 gene and its protein expression with the phosphorylation of the Rb protein in canine lymphoma cell lines.

When the p16 gene expression was high, the p16 protein was exhibited, and the phosphorylated pRb was barely identified (A), in contrast, when the p16 gene expression was low, the expression of the p16 protein was not observed, and the phosphorylated pRb was clearly detected (B).

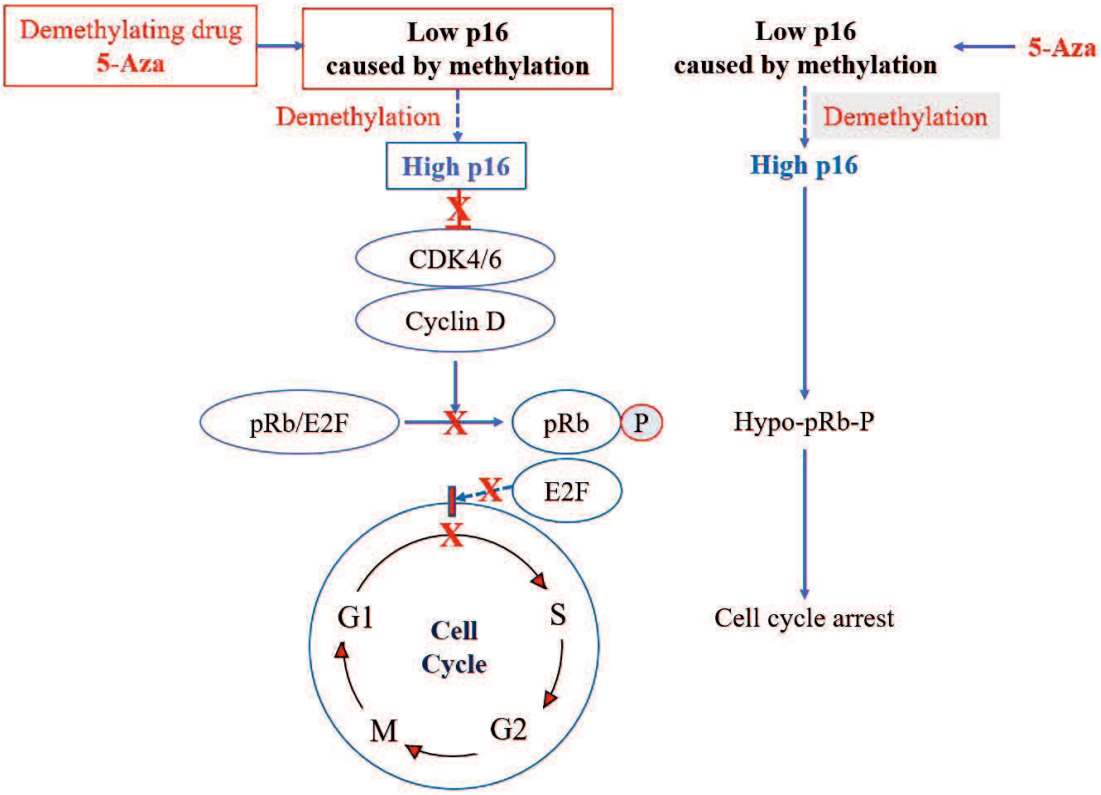


Fig. 10. The relationship between the inactivation of the p16 gene and its protein expression with the methylation of that gene, as well as hyperphosphorylation of the Rb protein in canine lymphoma cell lines.

The expression levels of the p16 gene and protein were significantly increased and phosphorylated pRb was decreased after demethylating treatment in low p16 cells.

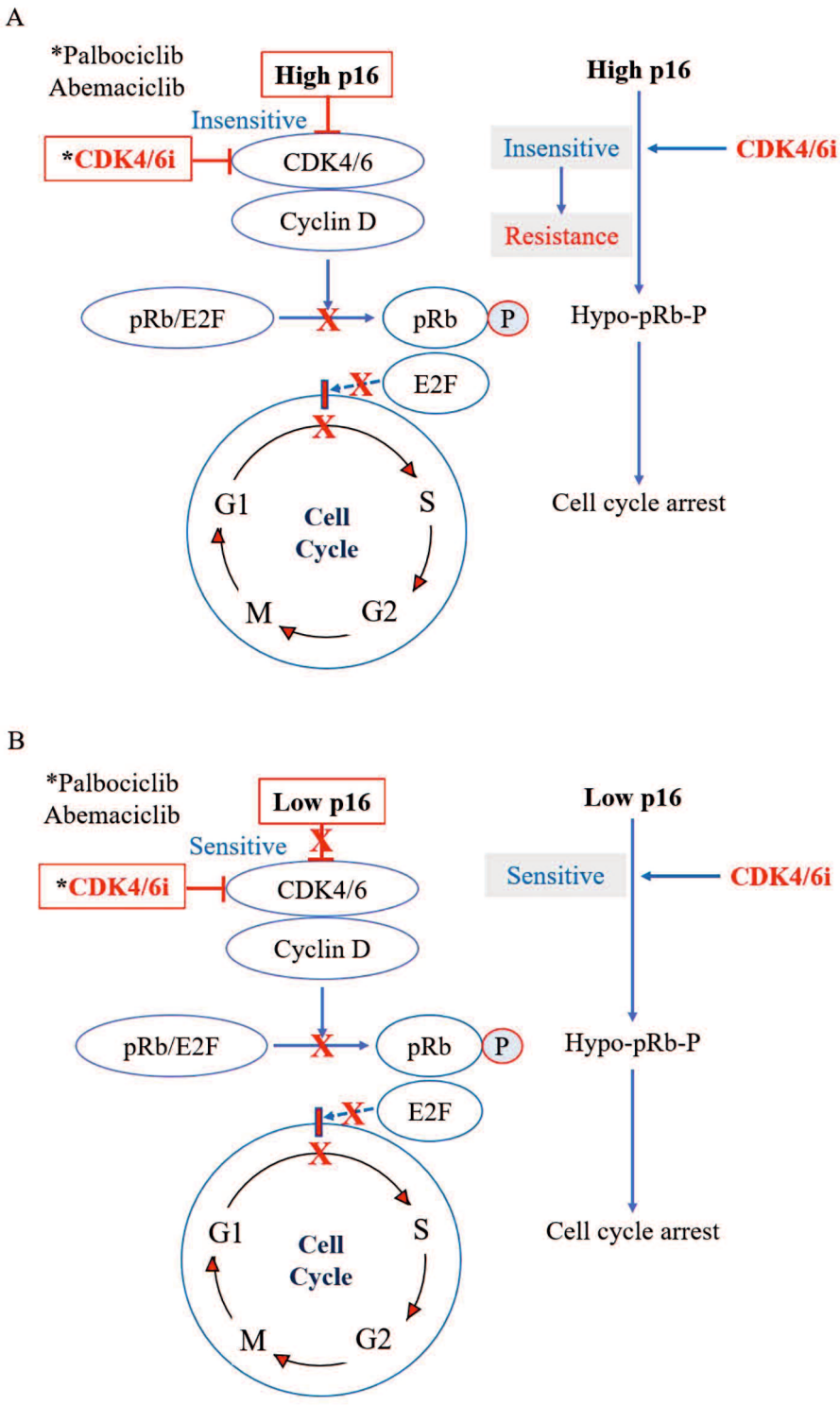


Fig. 11. The relationship between CDK4/6 inhibitors (palbociclib and abemaciclib) sensitivity to the various expression levels of the p16 protein and phosphorylated pRb in canine lymphoma cell lines.

The low p16 cells were sensitive (A) and the high p16 cells were relatively resistant (B) to CDK4/6 inhibitors.