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探討 YKL-40 作用於犬隻淋巴瘤細胞的生物學功能

To investigate the biological functions of YKL-40 on canine lymphoma cells

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中文摘要

YKL-40 是一種分泌型的醣蛋白，其生物學功能尚未完全清楚，可能參與了血管生成和細胞外基質重塑，且高血清 YKL-40 量已經在多種人類炎症性疾病以及惡性腫瘤中被發現。以往的研究還顯示出，在癌症患者中，高血清 YKL-40 量在不同類型癌症中，有潛力做為與疾病預後不良和短生存時間有關的生物指標，因此，瞭解 YKL-40 在癌症中的作用機制非常重要。然而，大部分 YKL-40 於癌症的研究都在人類實體腫瘤進行，對於犬癌症的研究極少；我們先前的研究發現，罹患癌症犬隻的血清 YKL-40 顯著高於健康犬，且在經過治療後復發的淋巴瘤犬血清中 YKL-40 高於未復發的犬隻。為了研究 YKL-40 在犬癌症中的生物學作用，我們在本研究中純化了重組犬 YKL-40 (rcYKL-40)，並將其應用於犬淋巴瘤細胞株 CLBL-1 (B 細胞) 和 UL-1 (T 細胞)，以評估其對犬淋巴瘤細胞的生物學功能的影響及其背後可能的細胞內信號傳遞途徑。研究結果顯示，包括外周血液淋巴細胞、單核球細胞、單核球來源巨噬細胞及成熟的單核球來源樹突細胞在內的多種犬血液細胞均會表現 YKL-40，而未成熟的單核球來源樹突細胞和犬淋巴瘤細胞 CLBL-1 和 UL-1 則不表現；在細胞實驗中，rcYKL-40 促進了 UL-1 細胞的增殖和遷移能力，但僅增進了 CLBL-1 細胞的侵襲能力，我們推測這是由於 CLBL-1 原本就生長較快，rcYKL-40 無法更加促進其增殖與遷移的能力。此外，在細胞活力存活試驗中發現，化療藥物阿黴素可以有效抑制 CLBL-1 和 UL-1 的增殖，而給予 rcYKL-40 可以保護兩種細胞減少阿黴素的傷害，並提高細胞存活率。透過細胞內信號途徑分析結果可得知，經 rcYKL-40 處理後，CLBL-1 的 ERK 和 UL-1 的 AKT 磷酸化蛋白的活性均增加，未來可以更深入探索 YKL-40 作用的分子機制，以及將 YKL-40 作為治療犬淋巴瘤潛在標靶的治療策略。

關鍵字：YKL-40、犬淋巴瘤、阿黴素、生物學功能、細胞內信號途徑

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Abstract

YKL-40 is a glycoprotein with incompletely understood biological functions. It was suggested to be involved in angiogenesis and extracellular matrix remodeling. Increased serum concentrations of YKL-40 have been detected in various human inflammatory diseases and malignancies. Previous studies also showed elevated serum levels of YKL-40 in cancer patients could be a potential biomarker that is associated with poor prognosis and short survival time in different kinds of cancer. Therefore, it's important to clarify how YKL-40 plays in cancers. However, most of the YKL-40 studies on cancer are based on human solid tumors. There are few studies on canine cancers. Our previous studies found that the serum YKL-40 level in cancer dogs had a substantial elevation compared to healthy dogs. Additionally, serum YKL-40 level in lymphoma dogs who relapsed after treatment was higher than without relapse. To investigate the biological functions of YKL-40 in dog cancer, recombinant canine YKL-40 (rcYK-40) was purified and applied to canine lymphoma cells, CLBL-1 (B cell) and UL-1 (T cell), in this study. The results show various canine blood cells including peripheral blood lymphocytes, monocytes, monocyte-derived macrophages, and mature monocyte-derived dendritic cells express YKL-40, whereas immature monocyte-derived dendritic cells and both CLBL-1 and UL-1, do not. In the cellular assay, the rcYKL-40 promotes the abilities of proliferation and migration on UL-1 cells, while it

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only elicits the invasion ability of CLBL-1 cells. Besides, in viability assay, doxorubicin (DOX) effectively inhibits the proliferation of CLBL-1 and UL-1. However, the administration of rcYKL-40 protects both cells from DOX damage and increases cell survival. By treating with the rcYKL40, the phosphorylation activity of ERK in CLBL-1 and AKT in UL-1 increased. Co-treatment of DOX and rcYKL40 on UL-1, but not CLBL-1, the activity of both proteins was also increased. These imply the potential role of YKL-40 on cellular processes and DOX-induced damage in canine lymphoma. Further studies may explore the molecular mechanisms of YKL-40 and potential therapeutic strategies targeting YKL-40 in canine lymphoma treatment.

Keywords: YKL-40, canine lymphoma, doxorubicin, biological functions, intracellular signaling pathways

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Chapter 1. Background and Literature Reviews

1.1 YKL-40

YKL-40 is a chitinase-like glycoprotein that is encoded by the *CHI3L1* gene, and it lacks chitinase activity because of the substitution of glutamic acid with lysine in the catalytic domain. It contains 383 amino acids, where the first amino acids of N-the terminal are tyrosine, lysine, and leucine, and the molecular mass is 40 kDa, so it is named YKL-40. Depending on the different species and tissues where YKL-40 was found, it is also named several different names: human cartilage glycoprotein-39 (HC gp39) (Hakala et al., 1993), breast regressing protein 39 (brp-39) (Archer et al., 1994), 38-kDa heparin-binding glycoprotein (gp38k) (Shackelton et al., 1995), chitinase-3-like-1 (CHI3L1) (Rehli et al., 1997), chondrites (Harvey et al., 1998), and mammary gland protein with 40-kDa (MGP-40) (Mohanty et al., 2003).

YKL-40 is common in eukaryotes and is generally expressed by various cells. Elevated serum YKL-40 levels and abnormal YKL-40 expressions have been found in various human inflammatory disorders and malignancies (Libreros & Iragavarapu-Charyulu, 2015). Besides, According to these previous reports, YKL-40 is correlated with cancer development and progression, and it has been suggested to participate in many biological activities, including cell growth, proliferation, immune cells differentiation, cancer cells migration, invasion, angiogenesis, extracellular matrix

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remodeling, and cell survival (Chen et al., 2011; He et al., 2013; Jefri et al., 2015; Johansen et al., 2009; Junker et al., 2005; Nishikawa & Millis, 2003). Therefore, clearing the relevance between YKL-40 and canine cancers and investigating the biological functions of YKL-40 in canine cancer cells may be beneficial to develop novel therapeutic options or a useful prognostic biomarker.

1.1.1 Expressions and regulations of YKL-40

Following its initial detection in human osteosarcoma cell line MG63 culture medium (Johansen et al., 1992), subsequent investigations revealed the secretion of YKL-40 by various cell types, such as stem cells, chondrocytes, macrophages, hepatic stellate cells, neutrophils, fibroblast-like cells, bone cells, vascular smooth muscle cells, synoviocytes, endothelial cells (Zhao et al., 2020), and cancer cells (pancreatic, oesophageal, gastrointestinal, head and neck, kidney, leukemia, lymphoma, ovarian, germ cell, uterine, respiratory tract, and skin) (GenBank NCBI database).

Based on many studies of human solid tumors, YKL-40 is over-expressed in various cancers, including small-cell lung cancer (Junker et al., 2005), cervical adenocarcinoma (Mitsuhashi et al., 2009), colon cancer (Shao et al., 2009), breast cancer (Wan et al., 2017), glioblastoma (Francescone et al., 2011), and colorectal cancer (Kawada et al., 2012). Some studies found that some cytokines, growth factors, and extracellular matrix (ECM) factors affect the regulation of YKL-40 (Zhao et al., 2020).

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For example, YKL-40 mRNA expression is inhibited by IL-1β and TGF-β in human chondrocytes and cartilage explant cultures (Johansen et al., 2001). The synthesis of YKL-40 is enhanced by Th1 cytokine and IFN- γ, whereas it is suppressed by Th2 cytokine and IL-4 in activated macrophages (Kzhyshkowska et al., 2006). In addition, cytokines IL-6, IL-17, and IL-18 have been reported that can stimulate YKL-40 secret into freshly isolated chondrocytes (Recklies et al., 2002; Xing et al., 1998).

1.1.2 The prognostic value of YKL-40 in inflammatory diseases and cancers

Overexpression of YKL-40 has been observed in various human inflammatory diseases, such as skeletal disease (Vos et al., 2000), rheumatoid arthritis (Matsumoto & Tsurumoto, 2001), respiratory disease (Chupp et al., 2007), and cardiovascular disease (Michelsen et al., 2010) and also in malignancies, such as glioblastoma (Pelloski et al., 2005), colon cancer (Cintin et al., 1999), breast cancer (Jensen et al., 2003), cervical adenocarcinomas (Mitsuhashi et al., 2009), prostate cancer (Jeet et al., 2014), and renal cell cancer (Vaananen et al., 2017).

Many previous studies show high serum level of YKL-40 in cancer patients is associated with poor outcomes and low survival rates in different kinds of human cancer, including ovarian cancer (Dehn et al., 2003), multiple myeloma (Mylin et al., 2015), melanoma (Lugowska et al., 2015), lung cancer (Jefri et al., 2015), endometrial cancer (Kotowicz et al., 2017), and rectal cancer (Fuksiewicz et al., 2018). Especially, some

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studies indicate that elevation in serum YKL-40 level is a more reliable predictor for differentiating endometrial and ovarian cancer patients from normal than serum CA-125, a well-established biomarker in reproductive cancer (Diefenbach et al., 2007; Kahramanoglu et al., 2018). Therefore, these reports indicate that the changes in YKL-40 concentration in the serum of cancer patients may be a valuable prognostic indicator.

1.1.3 The correlation between YKL-40 and non-cancer diseases

YKL-40 has been implicated in the pathogenesis of non-cancer diseases because elevated serum YKL-40 levels were detected in several acute and chronic inflammations, including rheumatoid arthritis (Jafari-Nakhjavani et al., 2019), asthma (Chupp et al., 2007), liver fibrosis (Nojgaard et al., 2003), and diabetes (Rathcke et al., 2006). These reports indicate that elevated circulating level of YKL-40 is correlated with disease activity. However, the exact functions of YKL-40 in inflammatory disorders are still unknown.

It is considered that immune cells such as macrophages and neutrophils which are the major sources of YKL-40 are locally activated in the inflammatory process (Mizoguchi, 2006). Furthermore, increased YKL-40 levels possibly protect the inflamed tissues in response to acute injury or inflammation, since YKL-40 promotes cell survival in adverse micro-environments (Francescone et al., 2011). Previous research on asthma has reported that the circulating level of YKL-40 is correlated with

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asthma severity, the thickness of the subepithelial basement membrane, exacerbations, and pulmonary function (Konradsen et al., 2013). Accordingly, YKL-40 could be regarded as a supportive marker for predicting some disease progression.

1.1.4 The correlation between YKL-40 and cancers

YKL-40 has been reported that its elevated circulating level and overexpression in tissues are strongly correlated with reduced patient survival in multiple types of cancers, including gastric cancer (Itik et al., 2011), colon cancer (Johansen et al., 2015), renal carcinoma (Berntsen et al., 2008), melanoma (Schmidt et al., 2006), Hodgkin lymphoma (Biggar et al., 2008), acute myeloid leukemia (Bergmann et al., 2005), glioblastoma (Iwamoto et al., 2011), and endometrial cancer (Diefenbach et al., 2007). Many previous studies have implicated that YKL-40 may play a crucial role in cancers. Nevertheless, the exact biological functions of YKL-40 in cancers are still not well elucidated.

According to past reports, it has been suggested that it mediates malignant cell proliferation and differentiation, stimulates angiogenesis, cancer cell metastasis, and invasion, affects extracellular tissue remodeling, and protects cells from undergoing apoptosis (Johansen et al., 2006). The data from gastric cancer research demonstrated that YKL-40 not only positively correlates with gastric cancer invasion depth, lymph node status, and tumor staging, but also promotes gastric cancer cell proliferation,

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migration, and metastasis (Geng et al., 2018). A study of bladder cancer also shows the increase of YKL-40 is closely related to tumor pathological stage, tumor invasion, and lymph node metastasis (Hao, Chen, Xie, & Liu, 2021). YKL-40 has been found increased expression in human glioblastoma cell line U87 in response to diverse stress induction and protect cells from adverse microenvironments of cancers such as inflammation, hypoxia, and the immediate effects of radiotherapy and cytostatic chemotherapy (Junker et al., 2005).

Although most YKL-40 research is focused on human solid tumors and there are fewer studies on YKL-40 in hematopoietic malignancies, these reports suggest that YKL-40 is also involved in such malignancies. A study of cutaneous T cell lymphoma shows similar results to previous studies of solid tumors that elevated serum YKL-40 levels in patients are related to the disease severity and YKL-40 promotes the proliferation of cutaneous T cell lymphoma cell lines (Suzuki et al., 2020). Accumulating evidence indicates that YKL-40 plays a key role in tumor development in solid tumors and hematopoietic malignancies. This evidence determines the significance of YKL-40 in tumor development.

1.1.5 The possible mechanisms of YKL-40 in cancers

The clear molecular mechanisms of these biological functions of YKL-40 in cancers are still not fully known. To date, there are some molecules have been identified

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as YKL-40 receptors, including interleukin-13 receptor subunit alpha-2 (IL-13Rα2) (He et al., 2013), chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Zhou et al., 2015), transmembrane protein 219 (TMEM219) (Lee et al., 2016), galectin-3 (Zhou et al., 2018), and CD44 (Geng et al., 2018). It has been reported that YKL-40 can bind or interact with these receptors to form complexes on the cell surface to activate the downstream signal pathways (Zhao et al., 2020).

Following that, mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) signaling cascades are initiated, resulting in the activation of both extracellular signal-regulated kinase-1/2 (ERK1/2) and protein kinase B (AKT)-mediated signaling cascades, crucial mitogenic signaling pathways implicated in diverse cellular progress and exhibiting dysregulated activation in numerous human malignancies (Ling & Recklies, 2004; Recklies et al., 2002). According to the previous study, YKL-40 binding to CD44 activates ERK and AKT pathways and phosphorylating β-catenin, promoting gastric cancer cell progression (Geng et al., 2018). YKL-40 also interacts with IL-13Rα2 and triggers the activation of the following MAPK signaling pathway, leading to the upregulation of matrix metalloproteinase genes expression which promotes breast cancer cell metastasis (Chen et al., 2017).

To deeper investigate the molecular mechanistic basis of YKL-40, a study of diffuse large B cell lymphoma (DLBCL) demonstrates that knockdown of *CHI3L1* gene

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expression inhibits cancer cell proliferation and enhances cancer cell apoptosis by modulating the expressions of cyclinD1 and cyclinD2 which are associated with the cell cycle (Yang et al., 2021). Furthermore, a research team in lung cancer provides evidence of YKL-40 regulating tumor growth and metastasis through a signal transducer and activator of transcription 6 (STAT6)-dependent macrophage2 polarization in lung cancer by using anti-*CHI3L1*-humanized antibody they previously developed (Yu et al., 2022). The YKL-40 expression response is delayed approximately 24 to 72 hours after stimulation, indicating that the YKL-40 is a secondary response downstream of other mechanisms (Johansen et al., 2006).

1.2 Canine lymphoma

Canine lymphoma is one of dogs' most common types of neoplasia (Ito et al., 2014). It shares many similarities with non-Hodkin's lymphoma (NHL) in humans (Richards & Suter, 2015) and is more commonly diagnosed in middle-aged and older dogs. Canine lymphoma is considered a heterogeneous disease with various clinical signs. This is a diverse group of cancers that arise from the uncontrolled and pathologic clonal expansion of either B- or T-cell lymphocytes (Fan, 2003). It can be categorized into five main types according to the anatomic forms: multicentric, alimentary (gastrointestinal), mediastinal, extranodal, and cutaneous lymphoma. Among these subtypes, multicentric lymphoma is the most common type, accounting for

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approximately 80-85% of cases (Ponce et al., 2010).

Canine lymphoma commonly originates in lymph nodes and primary nodal involvement is more common than the extra-nodal disease. However, any organ may be affected, it can spread to other organs such as the liver, spleen, and bone marrow. The methods of diagnosis include morphological characteristics, clinical pathology, thoracic and abdominal radiographs, cytology, histology, immunophenotyping, flow cytometry, PCR for antigen receptor rearrangement (PARR) assay, and biomarkers (Zandvliet, 2016).

The standard therapy for canine lymphoma is doxorubicin-based chemotherapy because it is relatively simple and affordable. Multidrug chemotherapy combining cyclophosphamide (CYC), hydroxydaunorubicin (doxorubicin, DOX), oncovin (vincristine), and prednisone (CHOP) has been generally considered the most effective therapeutic option for lymphoma (Thamm, 2019). Although CHOP-based chemotherapy leads to a high response rate in dogs, most canine lymphoma eventually relapses and becomes resistant. Besides, dogs with high-grade T cell lymphoma (TCL) have a lower remission rate and poorer response following CHOP-based chemotherapy compared to B cell lymphoma (BCL) (Moore, 2016). Therefore, more novel treatments and more specific therapeutic options are needed.

1.2.1 Incidence and predilection

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Canine lymphoma is a prevalent hematopoietic malignancy in dogs, with an approximate occurrence rate of 20-100 cases per 100,000 dogs (Zandvliet, 2016) and accounts for 5-7% of all canine cancers and 83% of all hematological cancers (Boerkamp et al., 2014; Dorn et al., 1968). The incidence of lymphoma in dogs varies by breed, age, and geographical location. Some breeds, such as Boxers, Golden retrievers, and Basset hounds, are more susceptible to developing lymphoma than others (Dobson, 2013), and the incidence also generally increases with age (the mean age at diagnosis ranging from 6.3 to 7.7 years) (Teske, 1994). Moreover, according to a report, there was a higher incidence of canine lymphoma in females than in males (Sánchez et al., 2019), while some other studies have reported no sex predilection (Teske, 1994).

1.2.2 Clinical subclassifications

Depending on anatomic involvement, canine lymphoma can be subclassified into five stages according to the definition from World Health Organization (WHO): single lymph node involved (stage I), multiple lymph nodes involved in one region (stage II), multiple lymph nodes generalized involved (stage III), above stages involved with liver or spleen (stage IV), and with blood or bone marrow (stage V). Different clinical presentations have further described five main classifications of canine lymphoma: multicentric, gastrointestinal, mediastinal, extranodal, and cutaneous lymphoma.

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Multicentric lymphoma (ML) which affects the peripheral lymph nodes is the most common clinical anatomic form and accounts for 73.2% of all canine lymphoma cases (Vezzali et al., 2010). It is frequently detected at stages III and IV; gastrointestinal lymphoma (GIL) which affects dogs' gastrointestinal tract is rarer than multicentric form and accounts for 5-7% of all canine lymphoma cases (Patnaik et al., 1977). This form is more commonly of T-cell origin (Coyle & Steinberg, 2004). The symptoms of GIL are often misunderstood as general enteritis, but the diagnosis can be obtained sufficiently through endoscopic and full-thickness biopsies (Miura et al., 2004); mediastinal lymphoma is more frequent in younger dogs (Day et al., 1996), accounting for approximately 19.5% of all cases and is almost exclusively of T-cell origin (Fournel-Fleury et al., 2002). It typically causes thymus and mediastinal lymphadenopathy. Due to the mediastinal mass or pleural effusion, this form results in dyspnea so it is fatal quickly without treatment; extranodal lymphoma is the rarest form of canine lymphoma. It can exist out of lymph nodes and anywhere in the body, and it includes pulmonary, hepatic, renal, cutaneous, and central nervous system lymphoma.

Morphologically, canine lymphoma can also be classified into various subtypes based on histological and immunophenotypical features and the most common subtypes include DLBCL, T-cell lymphoma, follicular lymphoma, marginal zone lymphoma, Burkitt-like lymphoma (BL), mantle cell lymphoma (MCL) (Valli et al., 2011). The

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majority of all different types of canine lymphoma are B-cell originated, a smaller proportion is arisen from T-cell (±30%) and non-B-/non-T-cell lymphomas (<5%) (Zandvliet, 2016). For instance, DLBCL, B-cell lymphoma, represents the most common form of lymphoma in dogs with an aggressive clinical course and accounts for approximately 31% of all NHL in westeWesterntries (Martelli et al., 2013). Overall, canine lymphoma is a heterogeneous malignancy, different forms have different symptoms and diagnostic methods.

1.2.3 Diagnosis

Canine lymphoma is typically diagnosed through a combination of clinical examination (morphological characteristics and clinical pathology), imaging studies, cytological and histological examinations by biopsy of affected lymph nodes, immunophenotyping by flow cytometry, and PCR-based technology. Clinical biochemistry and hematological profile can show a wide range of abnormalities, such as anemia, anorexia, weight loss, and fever (Gavazza et al., 2008). Imaging is an important tool in diagnosing and staging canine lymphoma and includes radiography, ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). That is also useful for evaluating lymph node size, structure, and hepatic and splenic involvement (Ballegeer et al., 2013; Crabtree et al., 2010).

Cytological and histological examination also plays a role in canine lymphoma

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diagnosis with fine-needle aspiration (FNA) or biopsy of the affected lymph nodes. This method provides quick, more detailed information on the cellular architecture of lymph nodes and distinguishes different subtypes with a relatively non-invasive initial diagnosis of canine lymphoma. Immunophenotyping is a specialized test involving antibodies to identify the specific cell markers on the surface of the lymphoma cells. A study found that it provided the most accurate and reliable diagnosis of lymphoma, followed by cytological and histological diagnosis (Valli et al., 2011).

Determining whether the origin of cells in dogs with lymphoma is from B cells or T cells is crucial as it provides valuable predictive insights regarding response rates and survival durations. PARR has been frequently used for identifying clonal expansions of lymphocytes with amplifying canine T-cell receptor γ and immunoglobulin heavy chain CDR3 regions (Burnett et al., 2003). Biomarkers also have been used to diagnose, predict prognosis, and guide treatment decisions. Some previous studies have evaluated the serum levels of vascular endothelial growth factor (VEGF), matrix-metalloproteinase (MMP) (Aresu et al., 2014), thymidine kinase 1 (TK1) (Saellstrom et al., 2022), and monocyte chemotactic protein-1 (MCP-1) (Perry et al., 2011). Overall, these diagnostic methods should be used in combination to guide treatment strategies and improve outcomes for canine lymphoma.

1.2.4 Treatment options

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Conventional treatment for canine lymphoma is chemotherapy, DOX-based combination, or CHOP which has been predominantly considered the therapy of choice to date, optionally assisted with surgery or radiation therapy. Chemotherapy agents aim to shrink tumors and remission rather than complete cure, damaging different cell cycle stages depending on the specific drug used. For example, the mechanism of DOX is to bind with topoisomerase II which is an essential enzyme for DNA replication to interfere with DNA synthesis and prevent cancer cells from dividing (Nitiss, 2009); vincristine can irreversibly bind with microtubules which are crucial for cell division to disrupt cancer cell division in metaphase of the cell cycle (Below & J, 2023). Therefore, chemotherapy mainly affects cancer cells that actively replicate or divide.

The goal of treating low-grade lymphomas which slowly grow is to long-term control instead of complete remission, so the chemotherapy protocols used to treat these indolent lymphomas are generally less intensive than those used for high-grade lymphomas (Valli et al., 2006). High-grade lymphomas are more aggressive and required more intensive multiple chemotherapy protocols to achieve remission, including the CHOP protocol (Aresu et al., 2014). According to the evaluation of CHOP protocol for treating canine lymphoma in several previous studies, complete response (CR) ranges from 85% to 95%, median progression-free survival (MPFS) times are 5

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to 9 months, and median survival time (MST) are around 12 months. Approximately 20% to 25% of dogs can survive for 2 years after treatment (Thamm, 2019).

Unfortunately, most canine lymphomas will eventually relapse after treatments and become drug resistant. Besides, chemotherapy drugs can cause several side effects, including vomiting, diarrhea, weight loss, fatigue, and lethargy. Moreover, some chemotherapy, such as DOX, have been reported that can lead to long-term heart-related issues, such as cardiomyopathy (Rawat et al., 2021). Consequently, novel treatments and therapeutic targets are needed to improve these predicaments.

1.3 Oncogenic behavior of cancer cells

Cancer cells are characterized by the abilities of unregulated cell growth and division, leading to tumor formation. The oncogenic behaviors of cancer cells are driven by a variety of genetic and epigenetic changes that alter the normal functions of cells. The hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, resisting cell apoptosis (programmed cell death), inducing angiogenesis, enabling replicative immortality, and evading the immune system (Hanahan & Weinberg, 2011). These oncogenic behaviors and the complex interplay of and microenvironment contribute to cancer development and progression.

To analyze cancer development, several types of *in vitro* assays have been

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developed depending on the different biological capabilities of cancer cells, such as MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and CCK8 (Cell Counting Kit) assay for cell proliferation, wound healing and transwell migration assay for cell metastasis, transwell invasion assay for cell invasion.

1.3.1 Abnormal proliferation

Unrestricted cell proliferation plays a crucial role in cancer development and progression because of the mutations involved in the regulation of cell division. Accordingly, Assessing the proliferation of cancer cells *in vitro* can measure the rate and extent of cancer cell growth, monitor the effectiveness of the treatments, and can also gain insights into the underlying mechanisms of cancer growth, and identify potential targets for new cancer therapies (Feitelson et al., 2015).

The methods of analyzing cell proliferation can be divided into different classes: dye exclusion assay, DNA synthesis rate, cellular metabolic activity, proliferation marker assay, and variations of ATP concentration (Adan et al., 2016). The basic principle of all cell proliferation assays is to detect cell viability and measure cell number or the changes in cell division rate. The most reliable and accurate method of these assays is DNA synthesis-based assay. That is designed to analyze the amount of new DNA synthesis based on incorporating labeled nucleotides into DNA (Ligasova et al., 2023), such as BrdU. Metabolic activity-based cell proliferation assay can be

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analyzed by detecting color change resulting from tetrazolium salts. There are many commercial kits and the protocols are simple, such as MTT, WST-1 (water-soluble tetrazolium-1), and CCK8. Detecting specific proteins expressed in proliferating cells is another method, such as Ki67 and proliferating cell nuclear antigen (PCNA), which are expressed in the cell cycle (Jurikova et al., 2016). Cell proliferation can also be analyzed by detecting the presence of ATP only existing in living cells. ATP concentration in cancer cells can be measured by using a bioluminescence-based assay involving luciferase and luciferin (Lee et al., 2012).

1.3.2 Migration and Invasion

Cancer cells possess a broad spectrum of migration and invasion mechanisms that allows them to change position from the primary tumor to a distant organ (Friedl & Wolf, 2003). In cancer development and progression, this biological process can lead to cancer metastasis, the most frequent cause of death in patients (Yamaguchi et al., 2005). Hence understanding the pivotal steps in this process is crucial to fight against cancers.

Two main *in vitro* methods of assessing cell migratory behavior have been described: wound closure assay and transwell migration assay. The former is to measure the closed distance over time may reveal specific migration changes or an impaired migratory phenotype (Justus et al., 2014). However, a limitation of using wound closure

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assay is that this method can only be applied to the adhering cells but not suspension cells. The second method is the transwell migration assay which measures the cell number migrated through the pores of the transwell membrane to assess the capacity of cell motility to directionally respond to various chemo-attractants and treatments (Pijuan et al., 2019). Transwell migration assay can be further used to investigate cell invasion by adding a layer of extracellular matrix on the transwell membrane to mimic the process of extracellular matrix (ECM) invasion (Justus et al., 2014). Transwell invasion assay measures the invasive cell number which can degrade the matrix, move through the ECM layer, and adhere to the bottom of the membrane (Kramer et al., 2013).

1.3.3 Cell death resistance

The ability to evade apoptosis is the hallmark of cancers and also one of the major obstacles to cancer treatment, since most current therapies primarily act by activating cell apoptosis in cancer cells, such as chemotherapy. Apoptosis, or programmed cell death occurs normally during development and plays a role in tissue homeostasis (Elmore, 2007). Cancer is the result of too little apoptosis occurring so that malignant cells survive and uncontrollably proliferate. Hence, monitoring the response of cancer cells in culture after treatment with different stimuli is the first step to designing new strategies against cancers. The methods of cell viability assay are the same as proliferation assay because both aim to measure living cells, including the number of

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cellular divisions, DNA synthesis, metabolic activity, or ATP concentration.

1.4 Conclusion

Dogs are the most popular companion animals, so their health is becoming increasingly valued. However, as dogs have longer lifespans, there is a higher incidence of cancer among them. Lymphoma is one of the most prevalent canine cancer, the high rate of relapse and drug resistance is the major challenge for treatment. YKL-40, a generally expressed glycoprotein, has been considered an attractive potential marker associated with poor clinical outcomes in various human tumors, but many previous related studies mainly focus on human solid tumors. To develop a novel strategy for canine lymphoma treatment, finding the relationship between YKL-40 and canine lymphoma is the first step. Through in vitro assays, this study aims to find the effects and possible mechanisms of YKL-40 affecting canine lymphoma cells at the cellular level.

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Chapter 2 Introduction

With advances in veterinary treatment and diagnostics technologies, domestic dogs live longer and have higher incidences of cancer than before. According to a previous study, cancer is the most common cause of death or reason for euthanasia in domesticated dogs in Taiwan (Huang et al., 2017). Among various types of canine cancer, lymphoma is one of the most diagnosed malignancies accounting for 7–24% of all canine cancers (Moore et al., 2018). To combat this highly heterogeneous tumor, DOX-based multidrug chemotherapy has been the standard therapeutic program for many years. Most dogs with lymphoma experience remission after treatment. However, as reported by North Carolina State Veterinary Hospital, up to 95% of canine lymphoma have relapsed following the treatment and even develop resistance to the chemotherapy. Therefore, looking for an effective therapeutic target or a useful prognostic biomarker would be essential for the treatment of canine lymphoma.

YKL-40, a secreted glycoprotein, is named for its first three N-terminal amino acids, tyrosine (Y), lysine (K), and leucine (L), and its molecular weight, 40 kDa. Various cells generally express this protein, including macrophages, neutrophils, stem cells, endothelial cells, fibroblast-like cells, synoviocytes, and vascular smooth muscle cells (Zhao et al., 2020). It is associated with various human inflammatory disorders and malignancies such as rectal cancer (Fuksiewicz et al., 2018), pancreatic cancer

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(Kjaergaard et al., 2021), bladder cancer (Hao, Chen, Xie, & Liu, 2021), breast cancer (Wan et al., 2017), and lymphoma (Suzuki et al., 2020). Previous studies also show that elevated serum YKL-40 levels in cancer patients is a potential biomarker associated with a poor clinical outcome and low survival rate in different kinds of cancer (Bergmann et al., 2005; Suzuki et al., 2020; Wan et al., 2017). Although the biological and physiological functions of YKL-40 are not fully understood, it is generally considered to play an important role in cancer. According to many previous studies, YKL-40 promotes cancer cell proliferation (Chen et al., 2011; Geng et al., 2018; Suzuki et al., 2020), stimulates cancer cell metastasis and invasion (Hao, Chen, Xie, & Liu, 2021; Hao et al., 2017; Jeet et al., 2014; Jefri et al., 2015), and protects cancer cells from apoptosis (Junker et al., 2005). Nevertheless, most YKL-40 research is based on human solid tumors and there are few YKL-40 studies on canine cancer.

To understand the role of YKL-40 in canine cancers, revealing the relevance between YKL-40 and canine cancer is the priority. Our previous studies have found that the serum YKL-40 level in cancer dogs is significantly higher than that in healthy dogs. In addition, the cancer dogs with higher serum YKL-40 levels have a higher risk of tumor recurrence and metastasis compared to those with lower serum YKL-40 levels (Cheng et al., 2019). In that study, canine lymphoma was the most common cancer accounting for one-third of cases (30/89). According to further investigations, serum YKL-40 level

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in lymphoma dogs who relapsed after treatment is higher than those without relapse. These seem to imply the correlation between YKL-40 and canine lymphoma. Therefore, this study aims to investigate the alterations of biological functions and signalings elicited by YKL-40 in canine B (CLBL-1) and T (UL-1) lymphoma cells using recombinant canine YKL-40 (rcYK-40).

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Chapter 3. Materials and Methods

3.1 Cell cultures

The Balb/3T3 cells were applied in the recombinant canine YKL-40 (rcYKL-40) protein expression. The BALB/3T3 (ATCC®CCL-163.2™) cell line was from the American Type Culture Collection (ATCC). Canine-derived cancer and non-cancer cell lines were used in this study. The canine lymphoma cell line, CLBL-1 (Rutgen et al., 2010), was generously donated by Dr. Rütgen (University of Veterinary Medicine Vienna, Vienna, Austria); UL-1 and CLC (Umeki et al., 2013) were donated by Dr. Umeki (Yamaguchi University, Yoshida, Yamaguchi, Japan). The cell lines mentioned above were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Simply®, GeneDireX, Inc., USA), supplemented with 10% fetal bovine serum (FBS, Caisson, USA) and 1% antibiotics (Caisson, USA) at 37℃ in a humidified atmosphere of 5% CO_2.

3.2 Generation of canine peripheral blood lymphocytes, monocytes, macrophages, and dendritic cells

To test if canine peripheral blood cells express YKL-40, we first isolate canine peripheral blood mononuclear cells (PBMCs) from packed red blood cells (PRBC) which are from blood-donor dogs, the PRBC was diluted with triple-volume sterile 1X

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Dulbecco's phosphate-buffered saline (DPBS, Simply®, GeneDireX, Inc., USA) and layered by using Ficoll-Paque PLUS density (density: 1.077 g/mL; Cytiva®, USA) and according to the manufacturer's instructions. In brief, canine PBMCs which appeared as the white layer above the Ficoll layer were collected into new 50 mL tubes by gradient centrifugation at 430 xg for 30 min at 25℃. The remaining red blood cells in canine PBMCs were lysed by using 1X RBC Lysis Solution (GOAL, Bio®, Taiwan) and according to the manufacturer's instructions. The isolated canine PBMCs were washed with sterile 1X DPBS and then cultured 7×10^7 each 15 cm dish adding RPMI 1640, supplemented with 10% self-serum, and 1% antibiotics overnight for monocytes/macrophages adhering. On the next day, Non-adherent canine peripheral blood lymphocytes (PBLs) were collected and the 10% self-serum contained in the medium was changed into 10% FBS. To obtain canine macrophages, the non-adherent cells were removed with medium change every 3 days, and the adherent monocytes would differentiate into macrophages after 8 days and be harvested. To obtain canine dendritic cells (DCs), monocytes were cultured in RPMI 1640 supplemented with 10% FBS and 1% antibiotics but also treated with 800 IU/mL granulocyte/macrophage colony-stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN, USA), 500 IU/mL interleukin 4 (IL-4, R&D Systems, Minneapolis, MN, USA), and 25 ng/mL FMS-like tyrosine kinase 3 ligand (Flt3L, R&D Systems, Minneapolis, MN, USA).

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Fresh medium and cytokines were changed every 3 days. On day 6, the medium supplemented as above added lipopolysaccharide (LPS, Sigma Chemical Company, Missouri, USA), then mature DCs were harvested on day 9. Each morphology of the stage of canine peripheral blood-derived cells was identified by using Liu's Stain (Baso®, Taiwan).

3.3 Expression and purification of recombinant canine YKL-40 protein

To provide exogenous YKL-40 applied to further research, we purified rcYKL-40, and the protocol was developed from the previous study. The canine *YKL-40* gene was cloned into *pcDNA3.1/V5-His-TOPO* vector (Invitrogen®, CA, USA), to yield new plasmid *prcYKL-40*. This construct was transfected into BALB/3T3 cells and cultured with a selective medium that contained 400 μg/mL G418. After the transfection and selection, the BALB/3T3 transformant, CL2436, can stably and spontaneously express the rcYKL-40 protein. CL2436 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Cytiva®, USA), supplemented with 10% FBS, 1% antibiotics, and 400 μg/mL G418 until the cells covered approximately 60-70% of the growth surface. Then, to secrete the rcYKL-40 into the medium, FBS-free DMEM (contained 400 μg/mL G418) was changed to the new cultured medium instead. After that, the cultured medium was collected and concentrated at 3,400 xg with Centrifugal Tubes (D-Tube™

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Dialyzer Maxi, MWCO 12-14 kDa Merck®, USA), then purified by His-Taq affinity column (IMAC FF 5mL, Cytiva®, USA). After the purification, desalt the protein by using PD-10 desalting columns containing sephadex™ G-25 medium (Cytiva®, USA). Then, the protein was quantified with the Coomassie Plus – The Better Bradford Assay™ Kit (Thermo Scientific Pierce, USA) which followed the procedure suggested by the manufacturer. The bovine serum albumin (Shanmugam et al.) standard (2 mg/mL, Thermo Scientific Pierce, USA) was used to generate a standard curve. Each reaction was performed for triplication.

3.4 Total RNA extraction

To confirm YKL-40 expression using RT-PCR, the total RNA of canine lymphoma cell lines and canine peripheral blood-derived cells were extracted first. Collect 1×10^7 cells by centrifuge at 430 xg for 5 min and remove the supernatant. The total was extracted by using a FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit (FAVORGEN®, Vienna) and according to the manufacturer's instructions. In brief, the cell pellet was resuspended in 350 μL FARB Buffer and 3.5 μL β-Mercaptoethanol in a 1.5 mL microcentrifuge tube. Transfer the mixture into the Filter Column placed in a 2 mL collection tube, then centrifuge at 18,400 xg for 2 min. Collect carefully the supernatant to a new 1.5 mL tube and add the same volume of 70% RNase-free ethanol by vortexing, then transfer the mixture into the FARB Mini Column back to the

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Collection Tube. Wash the FARB Mini Columns with 500 μL Wash Buffer 1, then twice with 750 μL Wash Buffer 2. Each wash was followed by a centrifuge at 18,400 xg for 1 min and the last centrifuge at 18,400 xg for 1 min to dry the FARB Mini Columns, then elute the RNA with 40 μL RNase-free water at 18,400 xg for 1 min. The concentrations of RNA were measured by spectrophotometer (Implen Nanophotometer®, USA), then digested contaminating genomic DNA in RNA solutions by using DNase I, RNase Free (BIONOVAS®, Canada). According to the manufacturer's suggestion, mix 1 μg RNA solution with 1 μL DNase I solution and 1 μL 1X Reaction Buffer and make the volume up to 10 μL with RNase-free water. Incubate the mixture for 10 min at 25℃, then add 1 μL stop solution (20 mM EDTA, pH 8.0) and incubate for 10 min at 65 ℃.

3.5 RT-PCR

The total RNA without genomic DNA was reverse-transcribed into cDNA by using the GScript Reverse Transcriptase Kit (GeneDirex®, Inc. USA). Mix 5 μg total RNA with 1 μL Oligo(dT)$_{20}$ (50 μM), 1 μL 10 mM dNTP Mix, and Nuclease-free water to a final volume of 13 μL in a sterile microfuge tube. Heat for 5 min at 6addsddsn add 4 μL 5X RT Buffer, 1 μL 0.1M DTT, 1 μL GScript RTase (200 units/μL), and 1 μL RNase-free water in a final volume of 20 μL. Incubate the tubes for 45 min at a reaction temperature of 50℃, and inactivate the enzyme for 15 min at 70℃. The gene expression

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was quantified by measuring the image area using Image J software, followed by the subtraction of background values. The mRNA levels were normalized to those of the reference gene (*GAPDH*) and calculated as follows: the relative expressions = target gene/reference gene.

3.6 PCR

To detect the YKL-40 mRNA expression in canine lymphoma cell lines and canine peripheral blood cells, the PCR was set in 25 μL reaction mixtures, containing 300 ng cDNA, 12.5 μL 2X Taq DNA Polymerase 2X Master Mix RED (AMPLIQON®, Denmark), 1 μL each primer, and nuclease-free water, conducted by LabCycler Basic (SensoQuest®, Germany). The primer pair of sYKL-40-F (5'- CGA CAC CTG GAC TTC ATC AG-3') and sYKL-40-R (5'- CGC TCT CCT GGT CAT CAT AC-3') was designed by GenBank database with accession number: XM_547343 and applied to detect the mRNA expression of YKL-40; cIL13Rα2-F (GAG ATA AAA GTT AAT CCT CCT CAG GA) and cIL13Rα2-R (ATG GAC ACC CAT GCC AGG TTT CCA AGA GC) were applied to detect the mRNA expression of IL13Rα2 (Tang L., 2001). The primer pair of GAPDH-F (5'- ACC ACA GTC CAT GCC ATC A-3') and GAPDH-R (5'- TCC ACC ACC CTG TTG CTG TA-3') was used as the internal control. The PCR conducted 35 thermal cycles of denaturation for 30 sec at 95℃, annealing for 30 sec at 53℃, and extension for 30 sec at 72℃. An initial heat for 10 min at 95℃ has added

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before the thermal cycle and an extra extension for 10 min at 72℃ was added at the end of the thermal cycle. The cDNA of the Balb/3T3 cells and CL2436 cells were used respectively as the negative and the positive control of YKL-40 mRNA expression. The PCR products were identified by 3% agarose electrophoresis.

3.7 Genomic DNA extraction

To confirm whether the canine lymphoma cell lines are B- or T-cell lymphoma using PCR, the genomic DNA was extracted first. Canine lymphoma cell lines CLBL-1 and UL-1 were centrifuged at 430 xg for 5 min and the supernatant was discarded. The genomic DNA of canine lymphoma cell lines was extracted by using a DNeasy® Blood & Tissue Kit (QIAGEN®, DNeasy®) and according to the manufacturer's instructions. In brief, the cell pellet was resuspended in 200 μL PBS and transferred to a 1.5 mL tube. Twenty microliters of Proteinase K and 200 μL Buffer AL were added into the tube, then incubated for 10 min at 56℃. Add 200 μL 99% ethanol and transfer the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube, then centrifuge at 6,030 xg for 1 min. Wash the column with Buffer AW1 and AW2, and then elute the DNA with 20 uL nuclease-free water and incubate for 1 min. The concentrations of genomic DNA were measured by spectrophotometer (Implen Nanophotometer®, USA).

3.8 PCR for antigen receptor rearrangements (PARR)

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To confirm the canine lymphoma cell lines used in this study, the PCR was set in 25 μL reaction mixtures, containing 300 ng gDNA, 12.5 μL 2X Taq DNA Polymerase 2x Master Mix RED (AMPLIQON®, Denmark), 1 μL each primer, and nuclease-free water, conducted by LabCycler Basic (SensoQuest®, Germany). The primers were developed by Burnett RC et al and used as follows: Sigmf1 (5'- TTC CCC CTC ATC ACC TGT GA-3') paired with Srμ3 (5'- GGT TGT TGA TTG CAC TGA GG-3') was used as the internal control (Cμ); CB1 (5'- CAG CCT GAG AGC CGA GGA CAC-3') paired respectively with either CB2 (5'- TGA GGA GAC GGT GAC CAG GGT-3') or CB3 (5'- TGA GGA CAC AAA GAG TGA GG-3') were applied to identify amplification of the immunoglobulin heavy chain (IgH) genes; DPA (5'- CTG TTG KTG CAG AAR CTG GAG AAG-3') paired with DPB (5'- AAC CCT GAG AAT TGT GCC AGG AC-3') and DPC (5'- GAG TTA CTA TAA TCC TGG TAM CTT CTG-3') was applied to identify amplification of TCRγ genes. The PCR conducted 35 thermal cycles of denaturation for 8 sec at 94℃, annealing for 10 sec at 60℃, and extension for 15 sec at 72℃. An initial heat for 15 min at 94℃ was added before the thermal cycle. The PCR products were identified by 3% agarose electrophoresis.

3.9 Proliferation assay

To test if rcYKL-40 can promote canine lymphoma cell lines proliferation, the cell proliferation assay was conducted. CLBL-1 and UL-1 were seeded into the 96-well flat-

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bottom plates at a density of 4×10^4 cells/well and 4×10^3 cells/well in 150 μL RPMI 1640 medium (5% FBS and 1% antibiotics) containing various concentrations of rcYKL-40 protein (100 ng/mL, 300 ng/mL, or 600 ng/mL) respectively, and cultured for 24, 48, 72 hours at 37℃, 5% CO_2. Recombinant canine YKL-40 was supplied in medium each day. Cell proliferation was determined at 24, 48, and 72 hours by using a cell counting kit (CCK8, Elabscience®, USA). According to the manufacturer's instructions, add 15 μL CCK8 into each well of the 96-well plate which contains 150 μL medium, and further incubated for 8 hours at 37℃, 5% CO_2. At the end of the incubation period, the 96-well plate was measured at a wavelength of 450 nm by SpectraMax M5 multi-mode microplate readers (Molecular Devices, USA). Three biological replicates were used for each treatment.

3.10 Transwell migration assay

The transwell migration tested if rcYKL-40 can facilitate canine lymphoma cell lines migration. Transwell migration assays were conducted in the 24-well plate, cell culture inserts (Corning®, Falcon®, USA) with a transparent PET track-etched membrane (pore size 8.0 μm). 300 ng/mL of rcYKL-40 protein was added with 4×10^5 cells in 200 μL of RPMI with 2% FBS. This prepared insert was then placed into the 24-well plate containing 500 μL of RPMI with 2% FBS and incubated for 4 days at 37℃, 5% CO_2. Recombinant canine YKL-40 was supplied in medium each day. After

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incubation, remove the inserts from the 24-well plate and centrifuge the plate at 430 xg for 10 min, then carefully discard 200 μL supernatant medium in the lower chambers. Resuspend the migrated cells in the lower chamber with the 300 μL remaining medium and transfer each 100 μL medium into each well of a new 96-well plate. Cell migration ability was determined at 96 hours by using a cell counting kit (CCK8, Elabscience®, USA). According to the manufacturer's instructions, add 10 μL CCK8 into each well of the 96-well plate and further incubated for 8 hours at 37℃, 5% CO_2. At the end of the incubation period, the 96-well plate was measured at a wavelength of 450 nm by SpectraMax M5 multi-mode microplate readers (Molecular Devices, USA). The protocol of the transwell migration assay is shown in Figure 7A. Three biological replicates were used for each treatment.

3.11 Invasion assay

The invasion assay tested whether rcYKL-40 can facilitate canine lymphoma cell line invasion. Invasion assays were conducted in cell culture inserts (BD Falcon, USA) with a transparent PET track-etched membrane (pore size 8.0 μm). 50 μL of 1mg/mL Matrigel (Corning®, Matrigel® Matrix, USA) was added into each insert and incubated for 4 hours at 37℃ to form a gel as shown in Figure 7B. 300 ng/mL of rcYKL-40 was added with 4×10^5 cells in 200 μL of RPMI with 2% FBS. This prepared insert was then placed into the 24-well plate containing 500 μL of RPMI with 2% FBS and incubated

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for 4 days at 37℃, 5% CO_2. Recombinant canine YKL-40 was supplied in medium each day. After incubation, remove the inserts from the 24-well plate and centrifuge the plate at 430 xg for 10 min, then carefully discard 200 μL supernatant medium in the lower chambers. Resuspend the migrated cells in the lower chamber with the 300 μL remaining medium and transfer each 100 μL medium into each well of a new 96-well plate. Cell invasion ability was determined at 96 hours by using a cell counting kit (CCK8, Elabscience®, USA). According to the manufacturer's instructions, add 10 μL CCK8 into each well of the 96-well plate and further incubated for 8 hours at 37℃, 5% CO_2. At the end of the incubation period, the 96-well plate was measured at a wavelength of 450 nm by SpectraMax M5 multi-mode microplate readers (Molecular Devices, USA). Three biological replicates were used for each treatment.

3.12 Viability assay

Cell viability assay was conducted to test if rcYKL-40 can protect canine lymphoma cells from doxorubicin (DOX)-induced cell death. CLBL-1 and UL-1 were respectively seeded into the 96-well flat-bottom plates at a density of 4×10^4 cells/well and 4×10^3 cells/well in 150 μL RPMI 1640 medium (5% FBS and 1% antibiotics) containing various concentrations of DOX (Adriamycin®, Pfizer, Inc. USA) (0, 2×10^{-3}, 4×10^{-3}, 8×10^{-3}, 1×10^{-2}, 2×10^{-2}, and 4×10^{-2} μg/mL on CLBL-1; 0, 2×10^{-4}, 2×10^{-3}, 2×10^{-2}, 2×10^{-1}, 2×10^{-0} μg/mL on UL-1) and cultured for 24, 48, 72 hours at 37℃, 5% CO_2.

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Cell viability was measured after incubation by CCK8 (Elabscience®, USA). After determining the IC50 which the concentration of DOX at half of the cell viability, 300 ng/mL of rcYKL-40 was added with 4×10^4 cells of CLBL-1 and 4×10^3 cells of UL-1 in 150 μL of 5% FBS RPMI containing IC50 DOX (6×10^{-3} μg/mL on CLBL-1; 2×10^{-2} μg/mL on UL-1). Recombinant canine YKL-40 was supplied in medium each day. Cell viability was measured after incubation by CCK8 (Elabscience®, USA). According to the manufacturer's instructions, add 15 μL CCK8 into each well of the 96-well plate which contains 150 μL medium, and further incubated for 8 hours at 37℃, 5% CO_2. At the end of the incubation period, the 96-well plate was measured at a wavelength of 450 nm by SpectraMax M5 multi-mode microplate readers (Molecular Devices, USA). Three biological replicates were used for each treatment.

3.13 Cell signal pathway analysis

To further study the pathways related to biological functions in canine lymphoma cells that may be activated by rcYKL-40, the cell signal pathway was analyzed. CLBL-1 and UL-1 were distributed 1×10^7 into the 1.5 mL tubes and incubated in serum-free RPMI 1640 for 1-hour serum starvation at 37℃, then the cells were treated with 300 ng rcYKL-40 in serum-free RPMI 1640 for 1 hour and the cells treated with PBS were the control. Centrifuge the tubes at 430 xg for 5 min, discarded the supernatant medium, and wash the cell pellet with cold PBS. Cells were resuspended and lysed by using 100

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μL lysis buffer containing 95 μL 1X Protein Lysis Buffer (RIPA, GOAL Bio®, Taiwan), 1 μL proteinase inhibitor (BIONOVAS®, Canada), 2 μL phosphatase inhibitor (Sigma-Aldrich, MO, USA), 1 μL NA_3VO_4, and 1 μL 10% SDS. Incubate for 30 min on ice with vortex thoroughly every 10 min and centrifuge at 9,400 xg for 20 min, then transfer the supernatant to a new tube on ice. The protein concentrations of the cell lysates were measured by using SMART™ BCA Protein Assay Kit (iNtRON Biotechnology, Inc., Korea). In brief, prepare diluted Albumin (BSA) standards with final concentration at 0, 25, 125, 250, 500, 750, 1,000, and 1,500 μg/mL and prepare Working Solution by mixing Solution A with Solution B (50:1). Add 25 μL of each standard or sample replicate into a microplate well and mix with 200 μL Working Solution, then incubate for 30 min at 37℃. The microplate was measured at a wavelength of 562 nm by SpectraMax M5 multi-mode microplate readers (Molecular Devices, USA). Fifty μg of each total protein was applied to the western blot and the PVDF membranes were probed with

3.14 Western blot analysis

The proteins were boiled in 4X sample buffer for 10 min at 100℃ to reduce and denature, then loaded into the wells of the 10% SDS-PAGE and electrophoresed at 100V for 15 min and 140V for 1 hour. The proteins on SDS-PAGE were electrotransferred to a 0.45 μm-pore polyvinylidene difluoride (PVDF) membrane

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(Bio-rad, USA) at 300 mA by using a transfer buffer containing 25 mM Tris, 192 mM, glycine and 20% (v/v) methanol in transfer tank for 1.5 hours at 4°C. After transferring, the PVDF membrane was blocked in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% skim milk at room temperature for 1 hour. The membrane was washed by TBST three times, 10 min each time. Subsequently, the membrane was probed with specific primary antibodies in TBST containing 0.5% skim milk and following HRP-conjugated secondary antibodies probe. The target proteins were detected by using Immobilon Western Chemiluminescent HRP Substrate (Merck, Darmstadt, Germany) and pictured by Multi-function Gel Image System (MultiGel-21®, TOPBIO, Taiwan). To confirm the purification of rcYKL-40, the primary antibodies were used as follows: mouse anti-Histidine antibody (1:5,000, Abcam®, USA) and rabbit anti-YKL-40 antibody (1:5,000; #A3166, ABclonal®, USA). The secondary antibodies were used as follows: Goat Anti-Mouse IgG (H+L) (1:5,000; #A32727, Invitrogen®, USA) and Goat Anti-Rabbit IgG (H+L) (1:5,000; #E-AB-1003, Elabscience®, USA, Houston, Texas), respectively. To confirm cell signal pathway analysis, the primary antibodies were used as follows: rabbit anti-AKT (1:1,000; #9272, Cell Signaling®, USA), rabbit anti-phospho-AKT (Ser473) (1:1,000; #9271, Cell Signaling®, USA), rabbit anti-p44/42 MAPK (ERK1/2) (1:1,000, #4695, Cell Signaling®, USA), and rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)

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(1:2,000, #4370, Cell Signaling®, USA). Goat Anti-Rabbit IgG (H+L) (1:5,000; #E-AB-1003, Elabscience®, USA, Houston, Texas) was used as the secondary antibody.

3.15 Statistical analysis

Results were expressed as individual data or mean ± SD. Experiments were repeated at least three times with similar results. Analysis significance was performed by using the student's t-test (Microsoft Excel), and a P value < 0.05 was considered significant.

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Chapter 4. Results

4.1 Examination of YKL-40 expression in various canine cells

To test if the cells used in the study express YKL-40, all canine lymphoma cell lines and canine peripheral blood cells were examined by RT-PCR using theusing0 primers listed in Table 1. As shown in Figure 1A, YKL-40 expression can be found in canine peripheral blood lymphocytes (Ly), canine monocyte-derived macrophages (Mϕ), canine monocytes (Mo), and mature monocyte-derived dendritic cells (mDC), while immature monocyte-derived dendritic cells (iDC) and all three lymphoma cells including CLBL-1, UL-1, and CLC do not express YKL-40. The quantification results of YKL-40 relative expression levels were estimated using the Image Jsented in Figure 1B.

4.2 Preparation of recombinant canine YKL-40 protein

4.2.1 Purification of recombinant canine YKL-40 protein

The rcYKL-40 was secreted in the culture medium of CL2436 cells, and the cultured medium was collected and concentrated by centrifugal tubes. The concentrated culture medium (400 mL) was purified by the His-tag affinity column with 30 mM, 35 mM, and 40 mM imidazole as washing buffers, sequentially, and 5 mL 500 mM imidazole as elution buffer (each for 5 mL) eluting out the proteins from the column

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which is the final purified products with the molecular weight of 46.7 kDa. In the serial purification process, these flow-through solutions included culture medium through the concentration tubes (B), concentrated culture medium before purification (C), washing buffers (W1-W3), and elution buffers through the column (1-3) were collected and separated with 10% SDS polyacrylamide gel electrophoresis (Figure 2A). All proteins were presented in the concentrated culture medium before purification and a minor of which were presented in the culture medium through the concentration tubes. Most non-target proteins were washed out with the wash buffers flowing through the column; no rest was detected in the elution buffers through the column. Most the target protein was bound with the His-Tag affinity column and eluted.

4.2.2 Identification of purified recombinant canine YKL-40

The final purified protein was analyzed by western blot, and the PVDF membrane was probed by mouse anti-histidine antibody. The predicted band of rcYKL-40 was shown on the PVDF membrane with a molecular weight of 46.7 kDa (Figure 2B).

4.3 Detection of gene rearrangements in canine lymphoma cell lines

To confirm whether the canine lymphoma cell lines used in the study are B- or T-cell lymphoma, PCR for antigen receptor rearrangements (PARR) was used to assess clonal rearrangements of antigen receptor genes. CLBL-1 was found to have rearrangements in the immunoglobulin heavy-chain (major) gene, UL-1 was found to

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have rearrangements in the TCRγ gene, and CLC was not found to have any gene rearrangement (Figure 3). This result suggests that CLBL-1 is B-cell lymphoma, UL-1 is T-cell lymphoma, and CLC is non-T non-B cell lymphoma.

4.4 Proliferation assay

To test if the rcYKL-40 can stimulate cell proliferation, the effects of rcYKL-40 on canine lymphoma cell lines were evaluated. As assessed by the CCK8 assay, the proliferation rate doesn't show any significant increase after being treated with different concentrations of rcYKL-40 in CLBL-1 (Figure 4A), while it shows significant improvement by rcYKL-40 at 48 and 72 hours in UL-1 (Figure 4B). Besides, the result of UL-1 treated with rcYKL-40 seems to be in a time-dependent manner.

4.5 Transwell migration assay

The transwell migration assay was conducted to evaluate the migration ability of suspensive canine lymphoma cells influenced by rc YKL-40. The result of the migration rate shows there is no significant difference between the control group and the 300 ng/mL rcYKL-40 group in CLBL-1, while the cells treated with 300 ng/mL rcYKL-40 significantly increased migrant to the lower chamber in UL-1 (Figure 5). The average absorbance of 562 nm OD value in the lower chambers shows that both CLBL-1 and UL-1 have a basic migration ability that in PBS and rcYKL-40 groups are significantly higher than the blank group which is without any cells in the medium.

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4.6 Invasion assay

The invasion assay was conducted to evaluate the invasion ability through extracellular matrices of suspensive canine lymphoma cells influenced by rcYKL-40. The results of the invasion rate show that CLBL-1 invasion across the Matrigel into the lower chamber was increased by treating 300 ng/mL rcYKL-40, while they don't show any significant difference between the control group and the rcYKL-40 group in UL-1 (Figure 6). The average absorbance of 562 nm OD value results show that both CLBL-1 and UL-1 have weak invasion abilities, but there is still a significant difference between PBS/rcYKL-40 groups and the blank group which is without any cells in the medium.

4.7 Viability assay

To test if the rcYKL-40 can rescue cells from cell death by doxorubicin (DOX), the effects of rcYKL-40 on canine lymphoma cell lines treated with IC50 DOX were evaluated. The results show that the cell viability of both CLBL-1 and UL-1 after being treated with IC50 DOX declined, whereas rising again by treating 300 ng/mL rcYKL-40 (Figure 7). This condition can be observed especially in UL-1, it seems that rcYKL40 promotes cell survival in a time-dependent manner. The increase of cell viability in the rcYKL40 group at 72 hours is more significant than at 24 hours (Figure 7B).

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4.8 Cell signal pathway analysis

To understand the precise mechanisms of the proliferative and protective effects of YKL-40, the downstream of YKL-40 in CLBL-1 and UL-1 was further investigated. A representative picture from three independent experiments is shown (Figure 8). The results show that rcYKL-40 significantly increases the phosphorylation of ERK1/2 but not AKT in CLBL-1, whereas it increases the phosphorylation of AKT but not ERK1/2 in UL-1. To determine whether ERK1/2 and AKT phosphorylations are involved in the survival effect of YKL-40, both cell lines were treated with DOX and rcYKL-40. The results show that after treatment with DOX, p-ERK is significantly induced in CLBL-1 and p-AKT is significantly reduced in UL-1. As treated with DOX and rcYKL-40, the expressions of p-ERK and p-AKT are significantly inhibited in CLBL-1, while they are enhanced in UL-1.

4.9 Expression of YKL-40-related receptor

To test if YKL-40 affects canine lymphoma cells used in the study through Interleukin-13 receptor subunit alpha 2 (IL13Rα2), the mRNA of CLBL-1 and UL-1 was examined by the total RNA transcribed into cDNA, and performed by PCR. CLBL-1 and UL-1 (Figure 9) were tested by using the IL13Rα2 primers (Table 3). The result shows that all canine lymphoma cell lines don’t express IL13Rα2 mRNA.

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Chapter 5. Discussion

According to previous studies in our laboratory, higher serum YKL-40 levels in cancer dogs are linked to an elevated risk of tumor recurrence and metastasis compared to dogs with lower YKL-40 levels (Cheng et al., 2019). A significant elevation in serum YKL-40 level is also further observed in lymphoma dogs with relapsed following treatment, in comparison to those who remained in remission. Combining these findings, it is postulated that YKL-40 may facilitate the progression of canine lymphoma by modulating various cellular biological processes. To confirm this perspective, therefore, investigating the effects of YKL-40 on canine lymphoma cells is essential.

In this study, we performed our studies using CLBL-1, UL-1, and CLC canine lymphoma cell lines, which are commonly used in canine lymphoma research. CLBL-1 has been reported that it is originally established from a dog with diffuse large B-cell lymphoma (DLBCL) (Rutgen et al., 2010). UL-1 was established from the ascites of a dog which were diagnosed with renal lymphoma (Umeki et al., 2013). CLC was established from the ascites of a dog with multiple abdominal lymphadenopathy, splenomegaly, and pleural effusion (Umeki et al., 2013). The antigen receptor gene rearrangements of these three canine lymphoma cell lines were reconfirmed in this study. CLC was not used in subsequent biological function assays due to the inability

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to determine whether it was a B or T cell lymphoma.

YKL-40 is known to be expressed in various cell types, including cancer cells. In my study, canine peripheral blood-derived macrophages, lymphocytes, and monocytes expressing YKL-40 can be observed in Figure 1. This finding aligns with previous reports demonstrating the detection of YKL-40 in human lymphocytes, monocytes, and macrophages these immune cells, as well as the association of YKL-40 with inflammation and immune responses (Johansen et al., 2006; Kzhyshkowska et al., 2006; Lee et al., 2009). Furthermore, our findings revealed that YKL-40 expression was absent in iDCs, while mDCs exhibited high-level YKL-40 expression. In contrast to our results, it has been reported that YKL-40 is expressed during the differentiation and maturation of human DCs and increases over time (Di Rosa et al., 2016). Additionally, YKL-40 expression has been detected in human DLBCL cell lines and cutaneous T-cell lymphoma (CTCL) cell lines (Suzuki et al., 2020; Yang et al., 2021). Notably, we did not detect YKL-40 expression in any of the canine lymphoma cell lines examined. in human research.

The disparity observed between my study and previous reports may be attributed to several factors. Firstly, it can be attributed to interspecies differences, as most YKL-40 studies have focused on human samples, few studies focused on canine YKL-40, whereas the samples in my study were sourced from dogs. The difference in YKL-40

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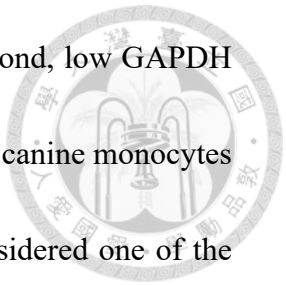

expression in cells from different species should be considered; second, low GAPDH expression which is used as an internal control was also observed in canine monocytes and iDC. Moreover, the poor quality of RNA samples is often considered one of the underestimated critical issues underlying gene expression results (Vermeulen et al., 2011) because that can negatively affect cDNA synthesis.

To improve RNA quality, proper sample collection and preservation are first required. In my study, these canine peripheral blood cell pallets are stored at -80℃ for no more than one week after separation. Reducing sample handling time, working on the ice, and using RNase-free techniques are also required to minimize RNA degradation. Another point worth noticing is that the presence of lymphocyte residues in both monocytes and iDCs was observed using Liu's stain. Thus, the purity of each sample should also be concerned and further optimized. Isolating adherent monocytes by washing the culture dishes with PBS to maximize the removal of suspended lymphocytes was adopted in my study, and the lymphocyte contamination diminished gradually during DC differentiation as well. A previous study has compared monocyte and macrophage purity and cell yield impaction of three commonly used monocyte isolation techniques, including plastic adhesion, negative selection, and CD14 positive selection (Nielsen et al., 2020). Although the plastic adhesion method is simple and inexpensive, the immune-based methods which present significantly higher monocytes

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yield and lower contamination with lymphocytes could be a concern.

YKL-40 possesses the capability to stimulate tumor cell proliferation, migration, and invasion, induce tissue remodeling, and facilitate angiogenesis through interactions with immune cells and tumor cells, ultimately contributing to tumor development (Schultz & Johansen, 2010). By our cellular assays as shown in Figures 4-7, enhanced cell proliferation and migration abilities were observed in UL-1 cells upon treatment with rcYKL-40, while increased cell invasion ability was observed in CLBL-1 cells. Additionally, rcYKL-40 conferred increased survival in both cell lines under doxorubicin (DOX) treatment.

YKL-40 has been implicated in promoting cell growth and division of various human tumors. In colorectal cancer, high expression of YKL-40 could promote the proliferation of colon cancer cells (Liu et al., 2020). Similarly, in prostate cancer, downregulation of YKL-40 expression has been shown to inhibit prostate cancer cell proliferation (Hao, Chen, Xie, Liu, et al., 2021); a research study on lung cancer utilizing recombinant YKL-40 demonstrated its direct stimulation of Lewis lung carcinoma (LLC) cell proliferation and growth (Yan et al., 2013). In my study, a time-dependent increase in the proliferation rates of UL-1 cells is observed, reaching statistical significance at 48 to 72 hours in the 100 and 300 ng/mL rcYKL-40 treatment groups. In contrast, no significant difference in proliferation rate was observed in

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CLBL-1. The significance in the 600 ng/mL group was not evident in the proliferation assay, and thus, the median concentration of 300 ng/mL was chosen for subsequent assays.

The role of YKL-40 in cancer cell migration has been extensively reported, highlighting its significant contribution to cancer progression. The addition of recombinant YKL-40 in the culture medium and transient expression of YKL-40 in cholangiocarcinoma (CCA) cell lines demonstrated enhanced tumor cell growth and migration (Thongsom et al., 2016). In breast cancer, YKL-40 has been reported to increase the migration of D492M which is a tumorigenic breast epithelial cell line (Morera et al., 2019). In a study of prostate cancer, a positive correlation was observed between YKL-40 expression and the migratory and invasive capabilities of prostate cells (Hao et al., 2017). In my study, the transwell migration rate was calculated as percentages using the lower chambers' average optical density (OD) values divided by those of the inserts. The absorbance averages of the lower chambers were also measured to confirm the presence of migrated cells. Both CLBL-1 and UL-1 exhibited baseline migration ability, with only UL-1 showing additional enhancement in the presence of rcYKL-40.

The enhanced effect of YKL-40 on cancer cell invasion has also been reported in different cancer and is considered a key role in cancer progression. YKL-40 which is

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secreted by M2 macrophages has been described to enhance metastasis of gastric and breast cancer cells by promoting cell migration and invasion in both cancer types (Chen et al., 2017). In my study, rcYKL-40 treatment leads to enhanced invasion of CLBL-1 cells, as assessed using the same calculation method as the migration rate. Conversely, no significant impact on the invasion rate of UL-1 cells is observed. However, both cell lines demonstrated weak invasion ability. To assess the effects of rcYKL-40 as a chemokine, adding rcYKL-40 in the lower chambers was further tested in this study. However, there is no significant difference between groups worth noticing in both migration and invasion assays.

By protecting cancer cells from apoptosis, YKL-40 can also contribute to tumor growth, progression, and resistance to therapy. The ability of YKL-40 to inhibit apoptosis can promote cell survival, allowing cancer cells to evade cell death signals and continue proliferating. The protective role of YKL-40 in inhibiting apoptosis has been reported in various cancer types. A study of glioblastoma shows that YKL-40 expression protects glioma cells from cisplatin-, etoposide- and doxorubicin-induced cell death (Ku et al., 2011). Knockdowning YKL-40 gene has been also found can promote the process of human DLBCL cell apoptosis (Yang et al., 2021). In my study, we utilized DOX to treat canine lymphoma cells in the viability assay, which is widely acknowledged as a primary chemotherapeutic agent for lymphoma treatment. The

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viability of canine lymphoma cells significantly decreased after damage by DOX, and the protective effect of rcYKL40 can be observed in both CLBL-1 and UL-1 cells. Furthermore, increased cell survival in a time-dependent manner can also be seen in UL-1.

To assess the effects of different rcYKL-40 concentrations, 100, 300, and 600 ng/mL rcYKL-40 which chose according to previous studies have been applied to proliferation assay in both cell lines. Various culture conditions were tested in the proliferation assay, and the significant difference between groups in UL-1 is only found in the medium containing 5% FBS, whereas no significant difference was observed in the serum-free medium. Studying the effects of specific factors or treatments on cells may require a serum-free environment to avoid potential interactions or interferences from the unidentified components in serum (Liu et al., 2023). While in my study, it seems like cells without any essential nutrients from FBS could not be well stimulated by rcYKL-40. On the other hand, by giving 5% FBS, cells have the basic condition to proliferation so that UL-1 can be further promoted by treating rcYKL-40.

Overall, these in vitro results further support the role of YKL-40 in promoting canine lymphoma cell proliferation, migration, and invasion and highlight its potential significance in canine lymphoma development and progression across different cell types. Moreover, rcYKL-40 seems to affect the biological functions of specific canine

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lymphoma cells rather than generally stimulating them. Besides, the protective effect of rcYKL40 is more obvious than the above effects including proliferation, migration, and invasion. These results may be considered as the different YKL-40 specific receptors presenting on the different cell surfaces and leading to different intracellular downstream signaling patterns. Besides, YKL-40 might mainly promote lymphoma cell resistance to DOX-induced cell death rather than promote their growth to achieve cancer progression. The findings can be taken into consideration, YKL-40 is more like having auxiliary effects but is not a major factor in promoting cancer cell development. To gain a more comprehensive understanding of YKL-40, further investigation into the potential molecular mechanisms underlying its role in canine lymphoma is essential.

Several key signaling pathways involved in YKL-40 have been reported in different cancer types. An endometrial cancer study demonstrates that inhibiting YKL-40 results in angiogenesis inhibition and microvessel density decrease by VEGF/VEGFR2 and ERK1/2 signaling pathways (Chen et al., 2021). In glioma, YKL-40 contributes to cancer cell proliferation and survival through persistent activation of the NF-κB signaling pathway (Zhao et al., 2022). Gastric cancer research indicates that the activation of the AKT signaling pathway is implicated as a potential mechanism for the oncogenic function of YKL-40 (Bi et al., 2009). YKL-40 promotes glioblastoma through the activation of PI3K/AKT and JAK/STAT3 pathways (Wang et al., 2018).

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YKL-40 significantly increased the phosphorylation of ERK1/2 at 30 minutes but not AKT in the human CTCL cell line (Suzuki et al., 2020).

To elucidate the underlying mechanisms behind the biological effects of YKL-40, we conducted further investigations into the downstream signaling pathways in CLBL-1 and UL-1 cells. As shown in Figure 8, the results of my study have found that rcYKL-40 significantly enhances the p-ERK1/2 in CLBL-1, while it induces p-AKT in UL-1. CLBL-1 and UL-1 show different phosphorylation patterns. Besides, to assess the potential involvement of p-ERK1/2 and p-AKT in the observed survival of YKL-40, both cell lines were also subjected to co-treatment with DOX and rcYKL-40. The results show that DOX treatment leads to a significant induction of both p-ERK1/2 and p-AKT in CLBL-1, whereas results in a significant reduction of pAKT in UL-1. Interestingly, upon co-treatment with DOX and rcYKL-40, substantial inhibition of p-ERK and p-AKT expression is observed in CLBL-1, while an opposite result is shown in UL-1.

These observations indicate that the specific mechanisms of YKL-40 promote canine lymphoma progression can be different based on the different cell types. The cellular response of CLBL-1 to DNA damage and cellular stress may be arisen by DOX so that both ERK and AKT pathways were activated. The activation of the ERK pathways has been noticed to play a role in DOX-induced cell death (Kumari et al.,

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2017). Conversely, the enhancement of p-ERK and p-AKT in UL-1 following co-treatment implies a potential mechanism by which YKL-40 exerts protective effects.

Further investigations are warranted to elucidate the precise interplay between YKL-40 and DOX in both cell lines. The mechanisms of DOX involve its ability to intercalate into DNA, thereby impeding the topoisomerase-II's function and generating free radicals that damage both DNA, proteins, and mitochondria (Thorn et al., 2011). These mechanisms ultimately induce not only apoptosis but also autophagy, senescence, and necrosis, leading to cancer cell death and cell growth arrest (Meredith & Dass, 2016). Understanding these mechanisms will provide valuable insights into the intricate cellular processes underlying the proliferative and protective effects of YKL-40.

To further investigate the reason for the different results of the above biological function assays between CLBL-1 and UL-1, the expressions of YKL-40 specific receptors in these two cell lines were examed in this study. Thus far, several receptors for YKL-40 have been elucidated, including interleukin-13 receptor subunit alpha-2 (IL13Rα2), transmembrane protein 219 (TMTM219), galectin-3, chemoattractant receptor-homologous 2 (CRTH2), and CD44 (Zhao et al., 2020). IL13Rα2 is the most common YKL-40 receptor, however, this study has shown that both CLBL-1 and UL-1 don’t express IL13Rα2. It seems like YKL-40 may affect canine lymphoma cells by interacting with other receptors, which suggests that further investigations by future

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researchers are warranted.

In conclusion, this study has investigated various aspects related to the role of YKL-40 in canine lymphoma cells. The results demonstrated that rcYKL-40 which has been developed before may promote canine lymphoma cells proliferation, migration, and invasion through MAPK/ERK or PI3K/AKT pathways, and promote cell survival by evading DOX-induced cell death. The findings highlight the importance of considering the heterogeneity of lymphoma cells and their specific signaling pathways in understanding the functional implications of YKL-40. Future studies may further elucidate the molecular mechanisms underlying the observed effects and explore potential therapeutic strategies targeting YKL-40 in canine lymphoma treatment.

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Tables

Table 1. The primers used in this study

Gene symbol	Primer name	Primer sequences (5'-3')	Expected size
YKL-40	sYKL-40-F	CGA CAC CTG GAC TTC ATC AG	415 bp
	sYKL-40-R	CGC TCT CCT GGT CAT CAT AC	
GAPDH	GAPDH-F	ACC ACA GTC CAT GCC ATC A	452 bp
	GAPDH-R	TCC ACC ACC CTG TTG CTG TA	
Cμ	Sigmf1	TTC CCC CTC ATC ACC TGT GA	130 bp
	Srμ3	GGT TGT TGA TTG CAC TGA GG	
IgH major	CB1	CAG CCT GAG AGC CGA GGA CAC	120 bp
	CB2	TGA GGA GAC GGT GAC CAG GGT	
IgH minor	CB1	CAG CCT GAG AGC CGA GGA CAC	120 bp
	CB3	TGA GGA CAC AAA GAG TGA GG	
TCRγ	DPA	CTG TTG KTG CAG AAR CTG GAG AAG	130 bp
	DPB	AAC CCT GAG AAT TGT GCC AGG AC	
	DPC	GAG TTA CTA TAA TCC TGG TAM CTT CTG	
IL13Rα2	cIL13Rα2-F	GAG ATA AAA GTT AAT CCT CCT CAG GA	408 bp
	cIL13Rα2-R	ATG GAC ACC CAT GCC AGG TTT CCA AGA GC	

(Burnett RC et al., 2003; Tang L., 2001)

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Figures

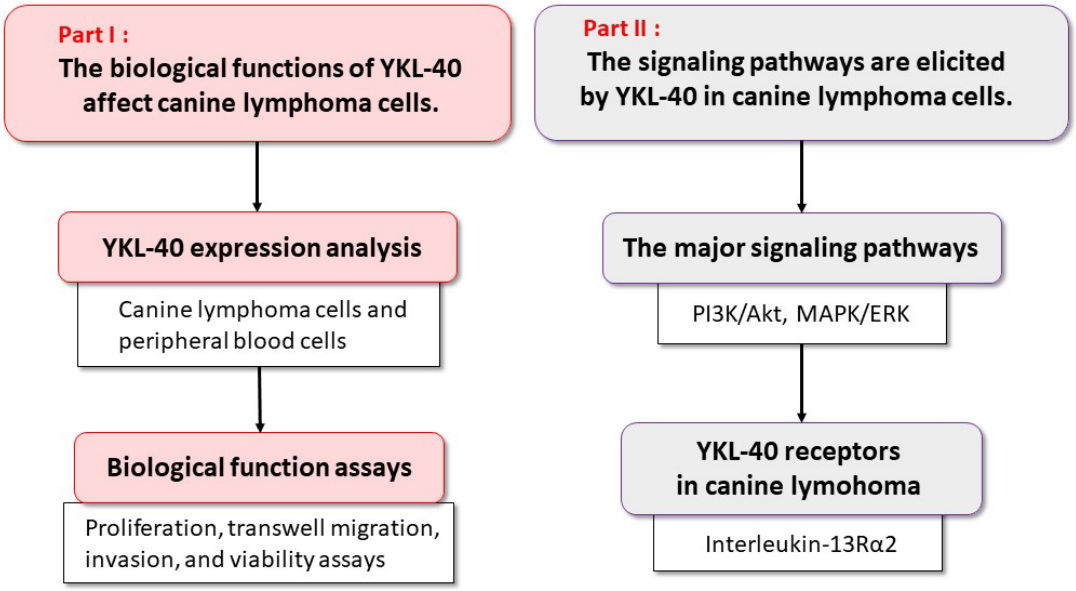

Figure 1. Experimental design

The experimental design was divided into two parts: the first part is to investigate the biological effects of rcYKL-40 on canine lymphoma cell lines, while the second part is to explore the potential underlying mechanisms behind these biological effects.

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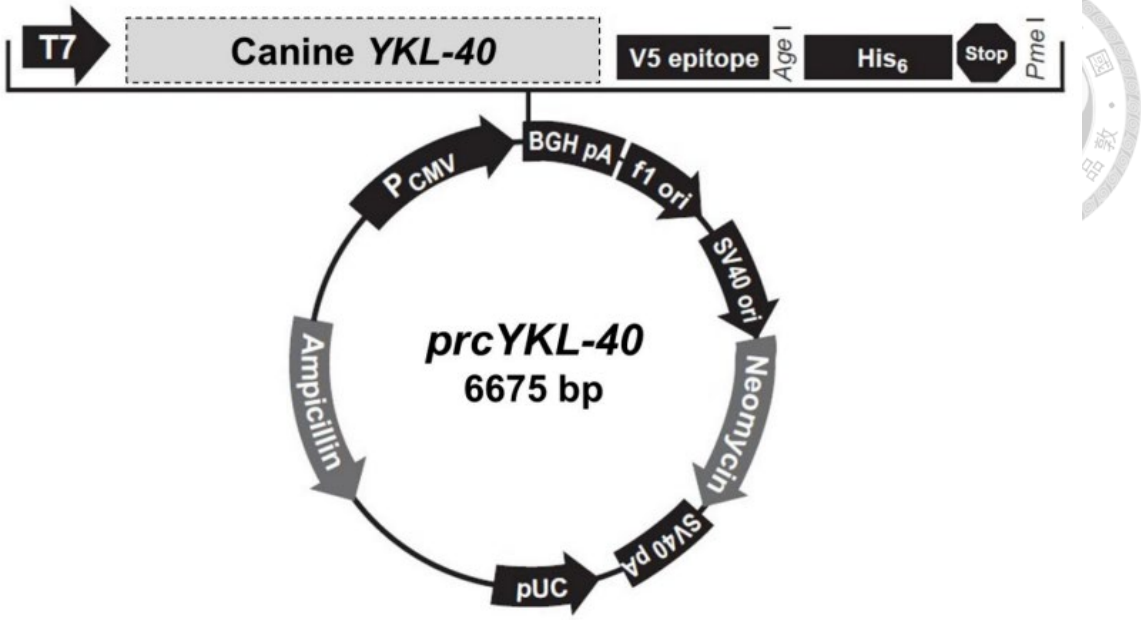

Figure 2. Schematic diagram of the construction of *prcYKL-40*

The canine YKL-40 gene was ligated into the vector pcDNA3.1/V5-HIS-TOPO to obtain *prcYKL-40*, which contains 6-histidine tag and neomycin phosphotransferase gene.

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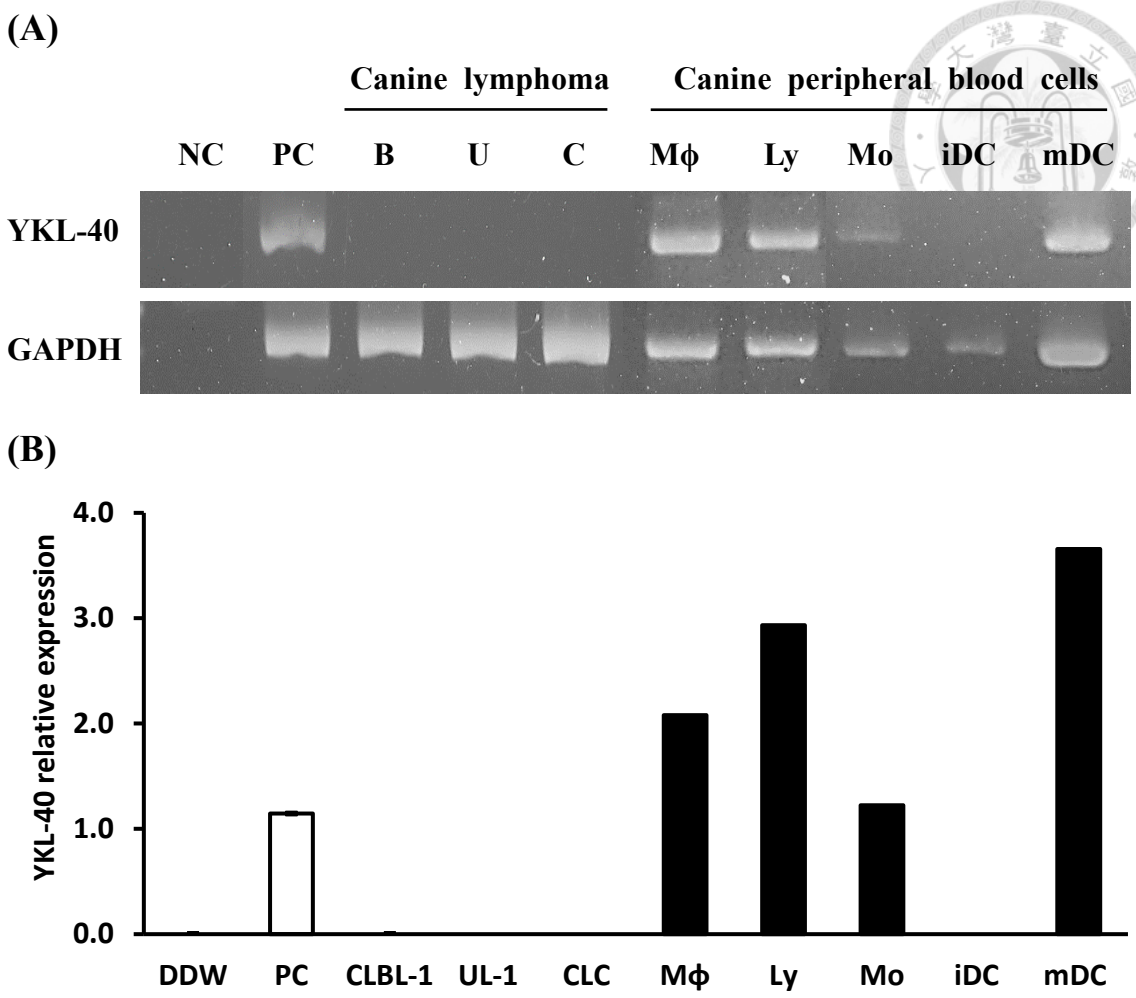

Figure 3. YKL-40 mRNA expressions of canine lymphoma cell lines and peripheral blood cells

(A) YKL-40 mRNA expressions of canine lymphoma cell lines and peripheral blood cells were detected by RT-PCR. (B) YKL-40 relative expressions were analyzed by Image J software. All of the canine lymphoma cell lines don't express YKL-40 mRNA, while canine peripheral blood cells all express YKL-40 mRNA except immature dendritic cells. NC: Negative control, PC: Positive control (CL2436), B: CLBL-1, U: UL-1, C: CLC, Mϕ: Macrophage, Ly: Lymphocyte, Mo: Monocyte, iDC: immature dendritic cell, mDC: mature dendritic cell.

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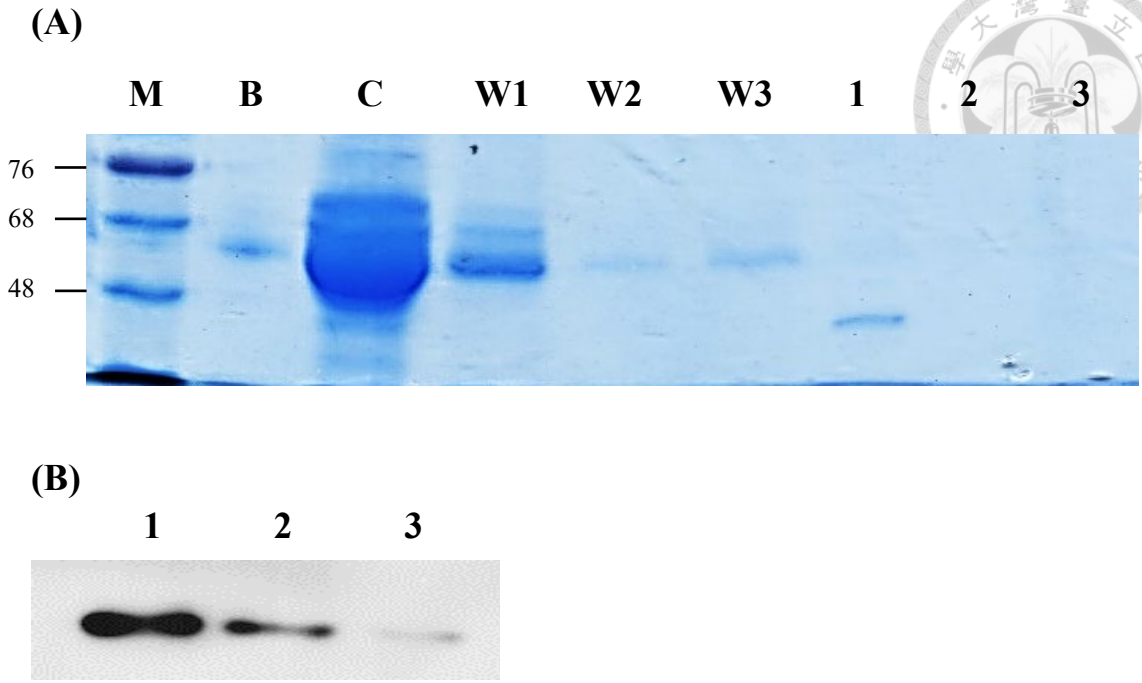

Figure 4. Purification of recombinant canine YKL-40 protein (46.7kDa)

The protein samples collected during the His-Taq purification were analyzed by (A) 10% SDS polyacrylamide gel electrophoresis and (B) Western blot which the membrane was probed with the mouse anti-Histidine antibody. B: Culture medium through the concentration tubes, C: Concentrated culture medium before purified, W1: Wash flow-through 1, W2: Wash flow-through 2, W3: Wash flow-through 3, 1: Elution flow-through 1, 2: Elution flow-through 2, 3: Elution flow-through 3. M: BlueRAY Prestained Protein Ladder (GeneDireX, Inc., USA).

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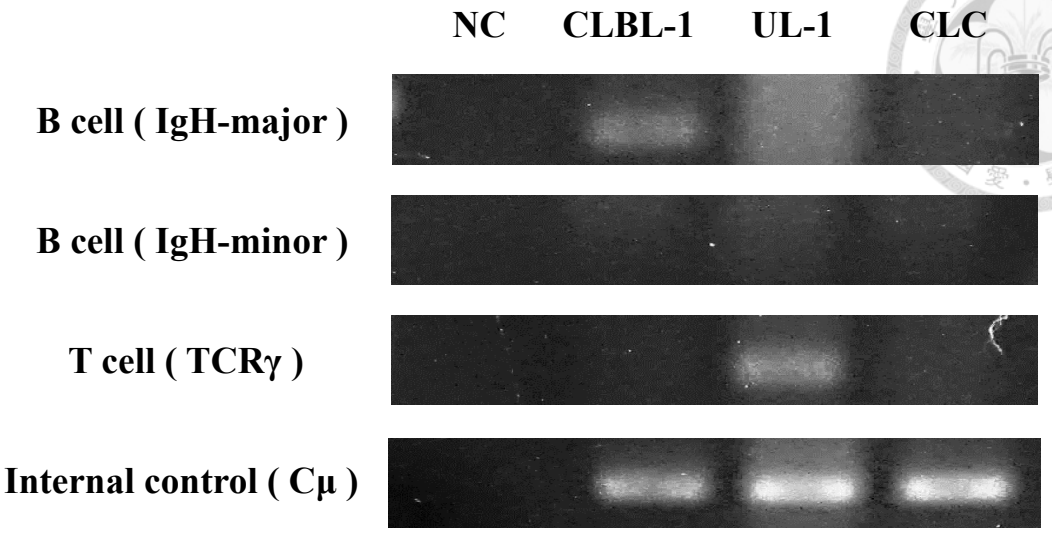

Figure 5. Detection of gene rearrangements in canine lymphoma cell lines

Rearrangements of antigen receptor genes in canine lymphoma cell lines were detected by PCR for antigen receptor rearrangement (PARR). IgH-major clonal rearrangement was detected in CLBL-1; TCRγ clonal rearrangement was detected in UL-1; none of IgH-major, IgH-minor, and TCRγ clonal rearrangement was detected in CLC. NC stands for negative control.

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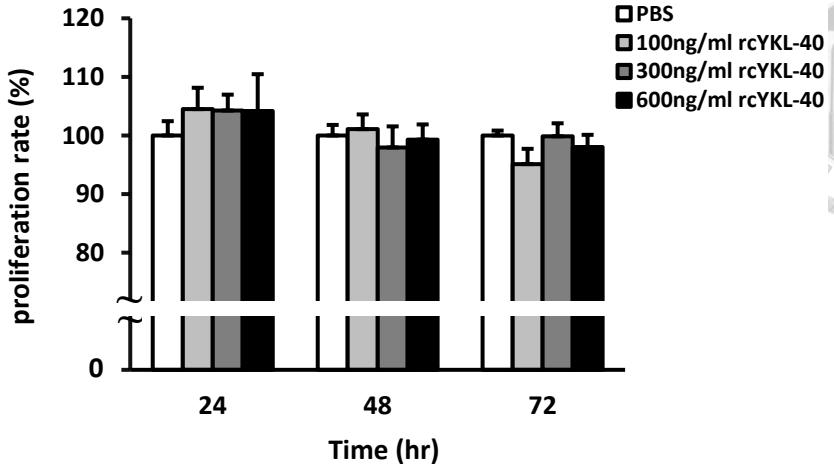
(A)



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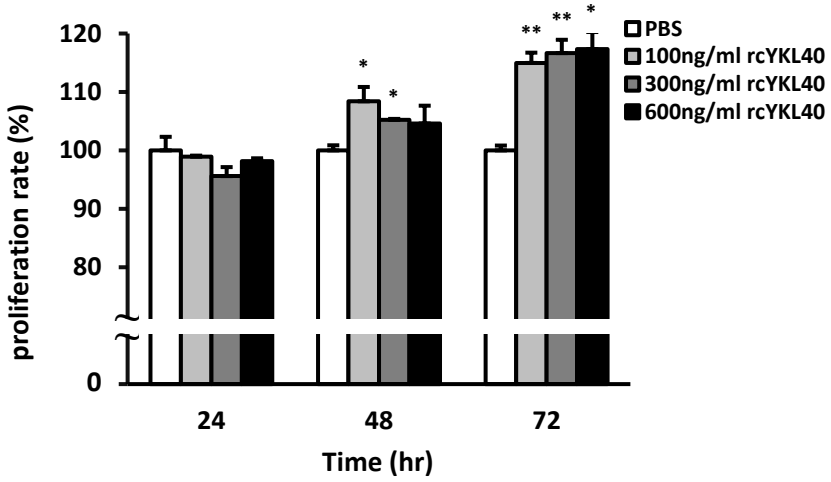

Figure 6. Effect of rcYKL-40 protein on cell proliferation rate

(A) CLBL-1 and (B) UL-1 were treated with 100, 300, and 600 ng/mL rcYKL-40 for cell proliferation assay. (A) The proliferation rate of CLBL-1 has no significant difference after being treated with rcYKL-40. (B) 100 and 300 ng/mL rcYKL-40 significantly increase the proliferation rate of UL-1 after 48 hours. *: p-value < 0.05, **: p-value < 0.01. The data shown are the mean ± SD (n=4).

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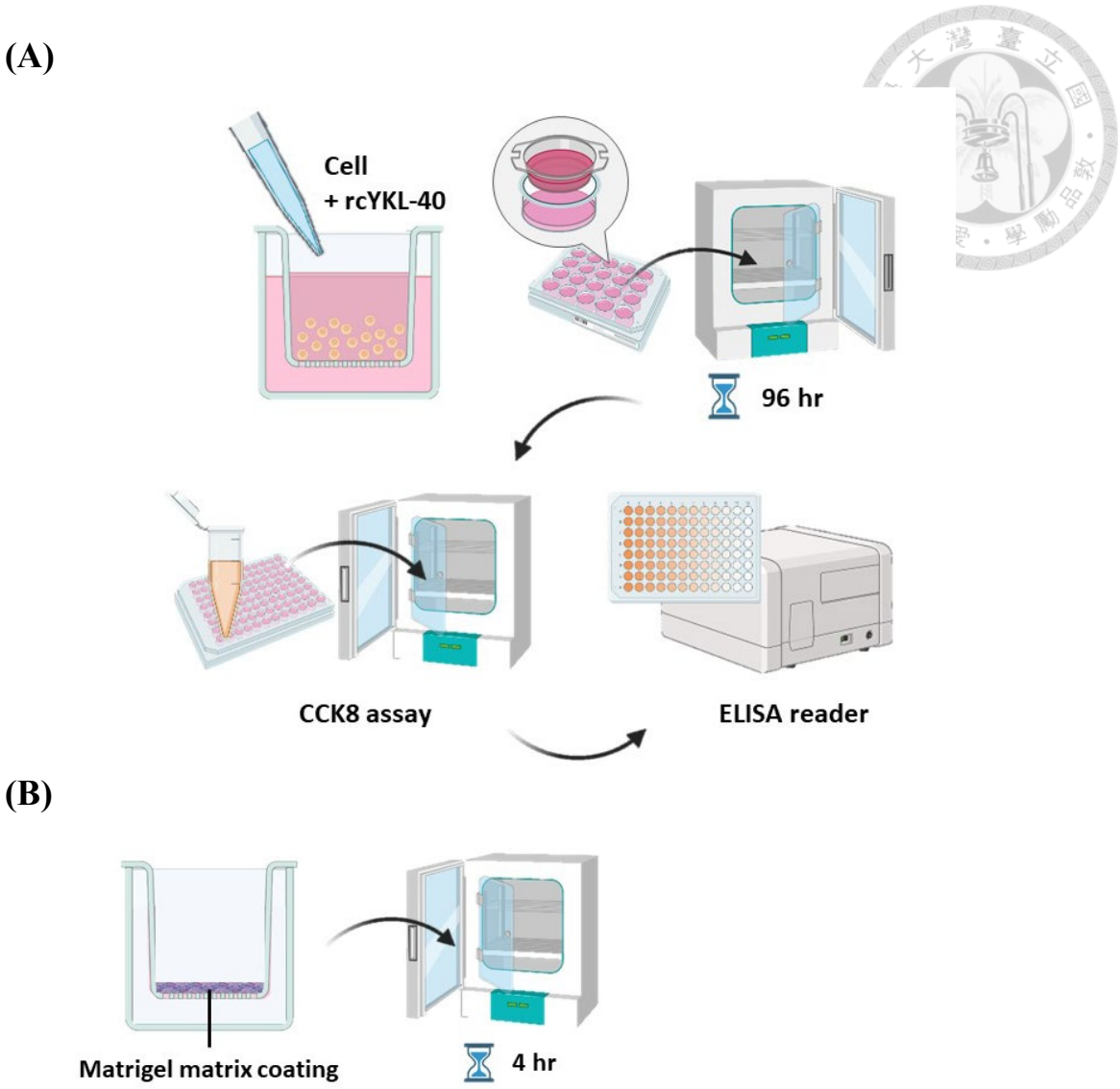

Figure 7. Transwell migration assay and invasion assay protocol

Transwell migration (A) and invasion (B) assays were performed using cell culture inserts in 12-well plates. CLBL-1 and UL-1 cells were seeded into the inserts and treated with rcYKL-40 or PBS. Subsequently, the cells were cultured for 96 hours, and their migration and invasion abilities were assessed using the CCK8 assay. The cell culture inserts for the invasion assay were pre-coated with Matrigel matrix (B).

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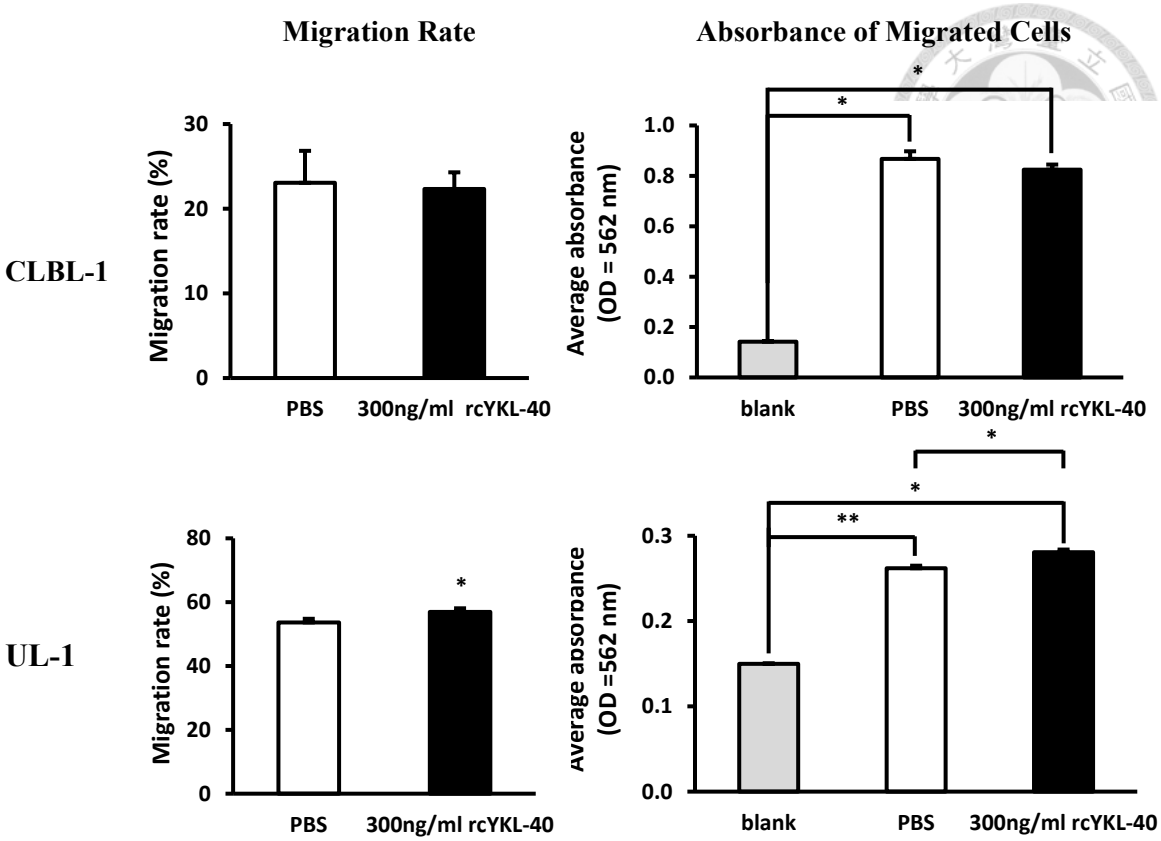

Figure 5. Effect of rcYKL-40 protein on cell migration

CLBL-1 and UL-1 were treated with 300 ng/mL rcYKL-40 for transwell migration assay. The migration rate of CLBL-1 has no significant difference after being treated with rcYKL-40. The average absorbance of the lower chambers shows that in both PBS and rcYKL-40 groups is significantly higher than the blank group, and rcYKL-40 has no additional effects on the migration ability of CLBL-1. 300 ng/mL rcYKL-40 significantly increases the migration rate of UL-1 at 96 hours. The result of average absorbance shows that the OD values of both PBS and rcYKL-40 group are significantly higher than the blank group, and that can be extra promoted by 300 ng/mL rcYKL-40. *: p-value < 0.05, **: p-value < 0.01. The data shown are the mean ± SD (n=4).

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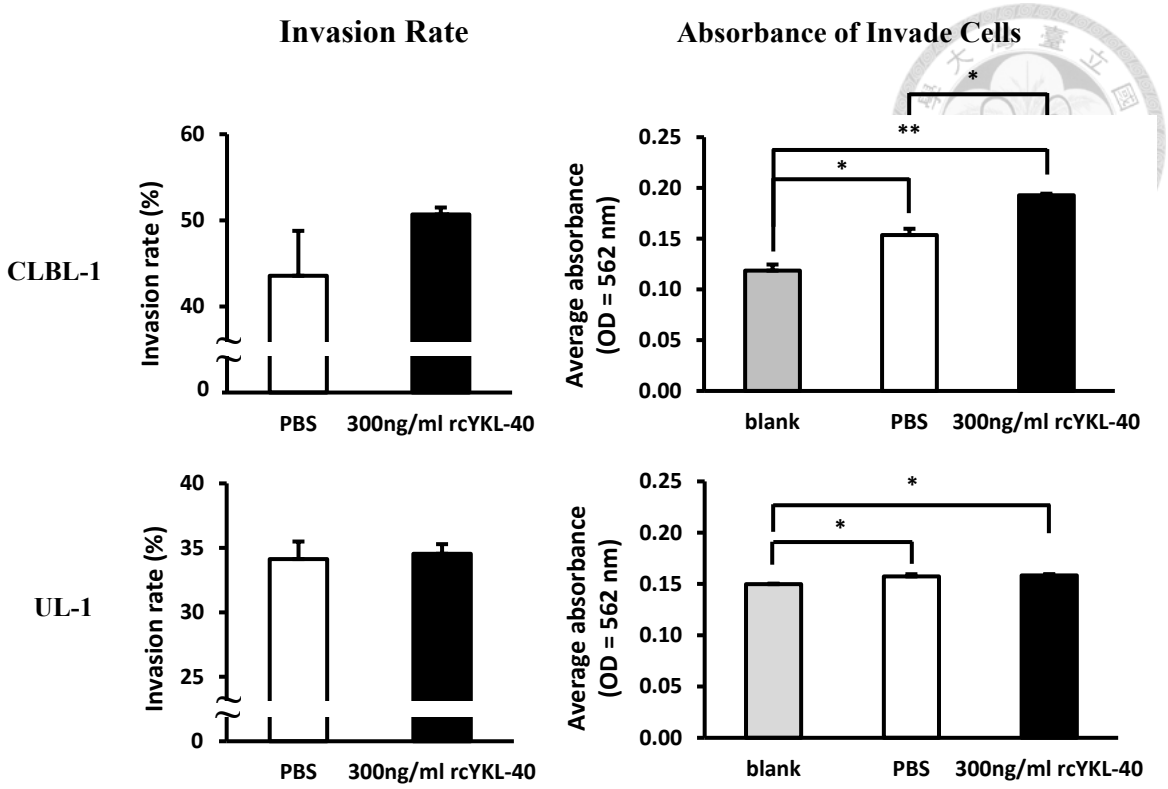

Figure 6. Effect of rcYKL-40 protein on cell invasion

CLBL-1 and UL-1 were treated with 300 ng/mL rcYKL-40 for transwell invasion assay. 300 ng/mL rcYKL-40 significantly increases the invasion rate of CLBL-1 at 96 hours. The average absorbance of the lower chambers in CLBL-1 shows that in both PBS and rcYKL-40 groups is significantly higher than the blank group, and rcYKL-40 has no additional effects on the migration ability of CLBL-1. The invasion rate of UL-1 has no significant difference after being treated with rcYKL-40. The average absorbance results of UL-1 also show that in PBS and rcYKL-40 groups is significantly higher than the blank group*: p-value < 0.05, **: p-value < 0.01. The data shown are the mean ± SD (n=4).

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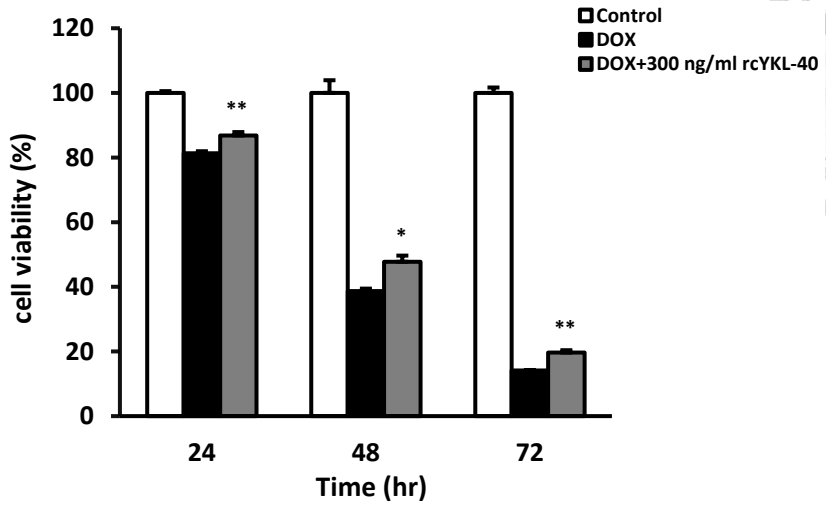
(A)



(B)

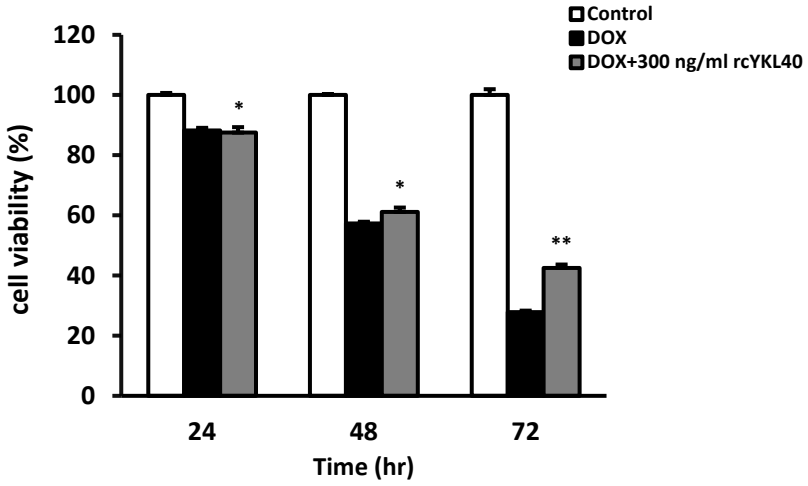


Figure 7. Effect of rcYKL-40 protein on cell viability

(A) CLBL-1 and (B) UL-1 were treated with 300 ng/mL rcYKL-40 at doxorubicin (DOX) half-maximal inhibition concentrations (IC50) for cell viability assay. (A) 300 ng/mL rcYKL-40 significantly increases the cell viability of CLBL-1 treated with DOX. (B) UL-1 treated with 300 ng/mL rcYKL-40 and DOX has significantly higher cell viability than treated with DOX only and in a time-dependent manner. *: p-value < 0.05, **: p-value < 0.01. The data shown are the mean ± SD (n=4).

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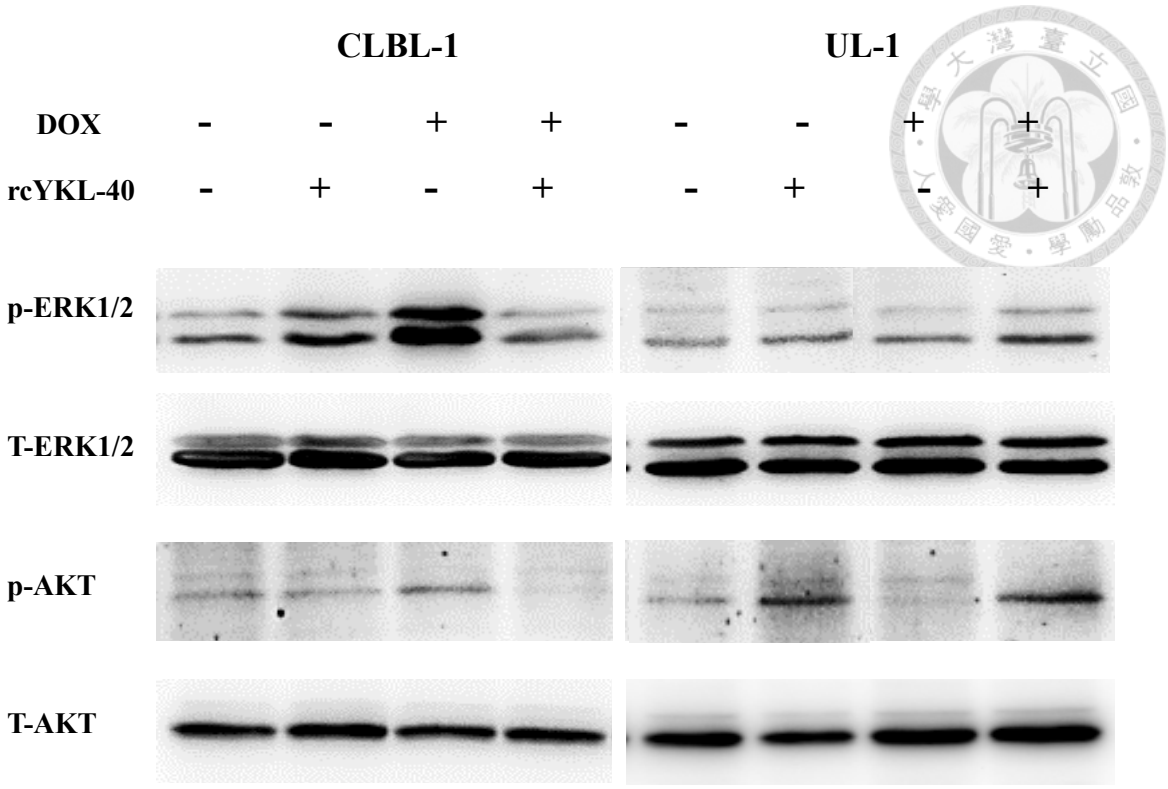

Figure 8. Cell signal pathway under rcYKL-40 and doxorubicin treatment

CLBL-1 and UL-1 were cultured with 300 ng/mL rcYKL-40 and IC50 DOX for 3 hours, and phospho-p44/42 MAPK (p-ERK1/2), phospho-AKT (p-AKT), total-p44/42 MAPK (T-ERK1/2), and total-AKT (T-AKT) were assessed by Western blotting. Recombinant canine YKL-40 significantly increases p-ERK1/2 expressions but not p-AKT expressions in CLBL-1, whereas it increases p-AKT expressions but not p-ERK1/2 expressions in UL-1. Phosphorylations of ERK1/2 and AKT are significantly reduced after co-treatment with rcYKL-40 and DOX in CLBL-1, and the expressions of p-ERK1/2 and p-AKT are significantly regained in UL-1. DOX, doxorubicin; AKT, protein kinase B; ERK, extracellular signal-regulated kinase 1/2.

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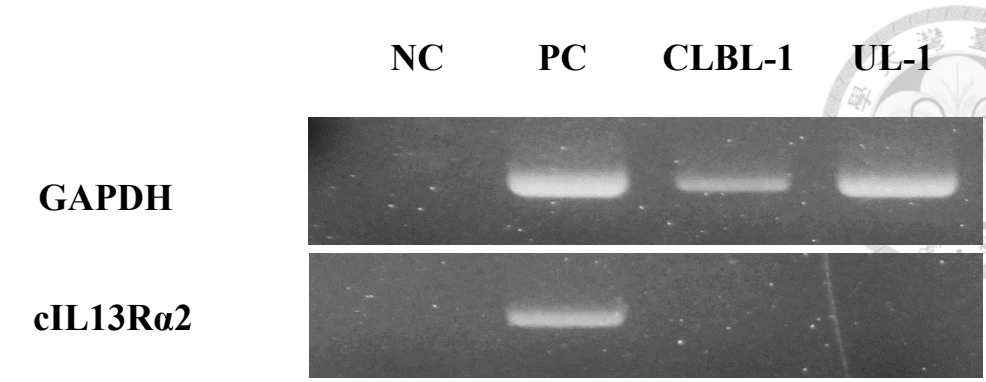

Figure 9. IL13Rα2 mRNA expressions of canine lymphoma cell lines

Interleukin-13 receptor subunit alpha 2 (IL13Rα2) mRNA expressions of canine lymphoma cell lines, CLBL-1 and UL-1, were detected by PCR. Both canine lymphoma cell lines don't express IL13Rα2 mRNA. NC: Negative control; PC: Positive control.

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