Studies on the pathophysiological role ofCD44 variant isoforms in canine lymphoma

博士論文(要約)

Studies on the pathophysiological role of CD44 variant isoforms in canine lymphoma

（犬のリンパ腫における CD44 variant isoform の病態生理学的役割に関する研究）

Tomoki MOTEGI

茂木　朋貴

CONTENTS

General Introduction

Lymphoma is one of the most common malignant tumors in dogs accounting for 7–24% of all canine tumors and approximately 83% of all hematopoietic tumors (Dobson et al., 2002). Canine lymphoma has been considered to be clinically heterogeneous disease resulting in various outcomes among patients. To predict the outcome, various prognostic factors have been reported in canine lymphoma, including body weight (Garrett et al., 2002), anatomic location (Withrow et al., 2013), histopathological classification (Valli et al., 2011), presence of anemia (Miller et al., 2009), clinical stage (Hosoya et al., 2007), clinical substage (Greenlee et al., 1990; Garrett et al., 2002), and immunophenotype (Greenlee et al., 1990). Among several subtypes based on anatomic form, immunophenotype, and cytological or histological characteristic (Marconato et al., 2013), multicentric B-cell high-grade lymphoma (diffuse large B-cell lymphoma) is the most common subtype in dogs (Fournel-Fleury et al., 1997; Vezzali et al., 2010).

Multicentric B-cell high-grade lymphoma respond favorably anticancer drug and combination chemotherapy is generally selected. CHOP-based protocol (C: cyclophosphamide, H: hydroxydaunorubicin [doxorubicin], O: oncovin [vincristine], P: prednisolone) is one of the most commonly used treatments for canine high grade B-cell lymphoma (Moore et al., 2001; Garrett et al., 2002; MacDonald et al., 2005; Simon et al., 2006; Hosoya et al., 2007; Burton et al., 2013; Curran and Thamm, 2016), resulting in complete remission in 70–90% of dogs with a disease-free period of 9–11 months. However, relapse is observed in most of the lymphoma dogs, when the disease control becomes difficult (Flory et al., 2011). As a result, the median survival time of dogs with multicentric B-cell high-grade lymphoma was reported to be 397 days in a study (Garrett et al., 2002).

The most common cause of the failure of treatment for canine lymphoma is the acquisition of drug resistance by tumor cells (Bergman et al., 2003). A number of factors associated with drug resistance have been studied in human and veterinary medicine

(Bergman et al., 2003; Lage, 2008). In canine lymphoma p-glycoprotein (Lee et al., 1996) and p53 (Dhaliwal et al., 2013) were reported to relate with drug resistance. Although p-glycoprotein was reported to enhance drug excretion from lymphoma cells (Lee et al., 1996), the expression is not common in dogs with drug resistant lymphoma (Tomiyasu et al., 2010). Mutant p53 also induces chemoresistance by avoiding apoptosis, but the mutation was not common in dogs with lymphoma (Koshino et al., 2016). In most of the canine lymphoma cases, alternative unknown molecules showed be contributing to drug resistance.On the other hand, some of the canine lymphoma cases showed limited sensitivity to anticancer drug from the beginning of the chemotherapy. These cases can be considered to possess natural drug resistance, through the mechanism is not clear.

In human studies, a number of molecules to contribute including drug resistance in untreated lymphoma cells have been identified such as CD44 (Stauder et al., 1995), p21 (Winter et al., 2010), CD5 (Ennishi et al., 2008), p53 (Sehn et al., 2005), and VEGFR2 (Gratzinger et al., 2010). Among a these molecules, I focused on CD44. CD44 is a hyaluronan-binding protein and has many physiological functions such as lymphocyte homing (Mackay et al., 1988), migration (Stoolman, 1989), cancer metastasis (Aruffo et al., 1990; Ponta et al., 2003), and cancer stem cell marker (Al-Hajj et al., 2003; Collins et al., 2005; Dalerba et al., 2007; Visvader and Lindeman, 2008). Various isoforms of CD44 generated through alternative mRNA splicing have been reported in human (Screaton et al., 1992). The standard form of human CD44 (CD44s), which consists of 10 exons, is expressed predominantly in hematopoietic cells and epithelial cells (Screaton et al., 1992). On the other hand, variant isoforms of CD44 (CD44v), which consists of 11 to 20 exons, with insertions of up to 10 exons at the membrane-proximal extracellular region, are expressed in many tissues such as epidermis, thyroid grand, tonsil, lymph node, and thymus in humans (Salles et al., 1993; Mackay et al., 1994). The physiological function of CD44v are not well understood.

Recent studies have shown that CD44v expression is related to the resistance to anticancer agents in many types of human tumors including mammary gland tumor (Van Pham et al., 2012), colorectal cancer (Ishimoto et al., 2011), and ovarian cancer (Gao et al., 2015). CD44v expression is also known to be a prognostic parameter in human non-Hodgkin's lymphoma (NHL) (Stauder et al., 1995) especially in diffuse large B-cell lymphoma (DLBCL) (Nagel et al., 2010; Wei et al., 2014).

In dogs, CD44 is also expressed in many tissues including macrophage, subset of lymphoma, epithelial cells, and thymus cells (Alldinger et al., 1999) as well as in some tumor cells such as mammary gland tumor (Paltian et al., 2009) and acute leukemia (Gelain et al., 2014). Moreover, some reports indicated that CD44 was a marker for cancer stem cell (Ferletta et al., 2011; Michishita et al., 2012) and poor prognosis (Magalhaes et al., 2013) in canine mammary gland tumor. Another study indicated analysis that *CD44* mRNA expression was related to tumor pathogenesis and prognostic importance in canine B-cell lymphoma by microarray (Zamani-Ahmadmahmudi et al., 2016). However, no report have distinguished CD44v from CD44s so far, and therefore the association between CD44v expression and prognosis remains unclear in canine tumors.

From these backgrounds, I conducted the investigations for the aims to identify the influence of CD44v expression on the prognosis in canine multicentric high-grade B-cell lymphoma. The regulatory mechanisms of drug resistance in canine lymphoma were also examined.

In chapter 1, I investigated the influence of the expression level of CD44 variant exons on the prognosis of dogs with multicentric high-grade B-cell lymphoma. Based on the results obtained in chapter 1, I studied the mechanism of doxorubicin and vincristine resistance in canine lymphoma expressing high level of CD44v in chapter 2. Further, gene expression profiles were compared between cases with CD44v high and low expression in

chapter 3. ESRP1 was found to regulate CD44v expression, and associated with poor prognosis in dogs with multicentric high-grade B-cell lymphoma.

Chapter 1

Influence of the expression of CD44 variant isoforms

on the prognosis in canine multicentric high-grade B-cell lymphoma

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Chapter 2

Characterization of CD44 variant isoforms in dogs and their association with drug resistance in canine lymphoma

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Chapter 3

Identification of ESRP1 as a regulator to induce

CD44 variant isoforms expression associated with clinical outcome

in dogs with high-grade B-cell lymphoma

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Conclusion

Drug resistance is one of the major causes of the failure of chemotherapy in lymphoma. Several mechanism have been proposed to confer drug resistance to tumor cells, however, molecules which contribute to drug resistance in large part of canine lymphoma cases remain unclear. The present thesis was carried out to elucidate the molecular mechanisms of drug resistance in canine lymphoma cells focusing on CD44v.

In chapter 1, the expression level of *CD44* variant exons 3, 6, and 7 were evaluated in dogs with multicentric high-grade B-cell lymphoma and compared with their prognosis. When the cut-off level was set at the mean minus 1 SD value calculated from normal lymph node samples, the OR rate, PFS, and OS were lower in the *CD44v*^{high} group than in the *CD44v*^{low} group. In particular, the *CD44v3*^{high} and *CD44v6*^{high} group showed lower OR rate and shorter PFS and OS compared to *CD44v3*^{high} and *CD44v6*^{high} group, respectively. Therefore, expression of these molecules (*CD44v3* and *CD44v6*) were expected to induce chemoresistance to the agents for CHOP.

To clarify the mechanism of drug resistance induced by *CD44v* in tumor cells, the CD44 variant isoforms predominantly expressed in canine lymphoma samples were transduced to canine lymphoma cell lines in chapter 2. The anticancer drug sensitivity was investigated using canine lymphoma cell lines overexpressing the most major CD44v isoforms, *CD44v3-5, 7* and *CD44v6*. The sensitivity to DXR and VCR were significantly decreased in CD44v6-overexpressed cells, while not changed in CD44v3-5, 7-overexpressed cells. Reduced drug sensitivity observed in CD44v6-overexpressed cells were possibly due to activation in Akt signaling since the sensitivity to DXR and VCR was recovered by Akt/PI3k inhibitor, LY249002.

In order to find a regulator of CD44 variant isoforms expression in canine lymphoma, comprehensive gene expression profiles was compared between cases with higher and lower expression of CD44v. ESRP1 was found to be highly expressed in cases with high

expression of *CD44v* mRNA. Cell line overexpressing ESRP1 showed increased level of CD44v together with reduced sensitivity to DXR and VCR. Moreover, expression of *ESRP1* was correlated with poor prognosis in dogs with multicentric high-grade B-cell lymphoma possibly by *CD44v* mRNA induction.

In conclusion, a part of pathophysiological role of CD44 variant isoforms was revealed in canine multicentric high-grade B-cell lymphoma. Higher expression of CD44 variant isoforms induced poor prognosis and reduced sensitivity to CHOP-treatment in canine multicentric high-grade B-cell lymphoma. ESRP1 was shown to induce higher expression of CD44v, possible by regulating the CD44 alternative splicing. Further, in lymphoma cases with higher expression of ESRP1, sensitivity to DXR and VCR decreased together with the activation of Akt signaling. Inhibition of Akt signaling or ESRP1 protein might be a new strategy of treatment in canine lymphoma with high expression of CD44v6. Acalabrutinib, a BTK inhibitor repressing p-Akt, has recently been reported to inhibit proliferation in a subset of canine DLBCL (Harrington et al., 2016). Lymphoma cases with higher CD44v6 expression might be a candidate target cases of Acalabrutinib. On the other hand, ESRP1 inhibitor has not been found at present, by compound screening using ESRP1-overexpressed cells, it might be possible to develop a novel treatment in dogs, which is applicable to human lymphoma. I believe that the results obtained in this thesis a part of the molecular mechanisms of natural drug resistance in canine lymphoma, and would be a help to establish novel treatment in human and canine lymphoma

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