Studies of *PTPN11*/SHP2 mutations in canine histiocytic sarcoma cells

Summary of Doctoral Thesis

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Canine histiocytic sarcoma (HS) is a highly metastatic and an aggressive neoplasm. Because of its aggressive nature, chemotherapeutic agents often have been used for the treatment of HS. Although clinical benefits have been demonstrated for chemotherapy in dogs with HS, this tumor remains fatal with short survival times. Therefore, new therapeutic strategy is needed for the treatment of HS in dogs.

Src homology 2 domain-containing phosphatase 2 (SHP2), which is encoded by *PTPN11*, is a non-receptor tyrosine phosphatase that positively regulates downstream signaling of various receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR), KIT, FLT-3, and platelet-derived growth factor receptor by direct interaction with auto-phosphorylated RTKs or by indirect interaction via binding to tyrosine-phosphorylated adaptor proteins. Recently, putative SHP2 mutations p.Glu76Lys and p.Gly503Val in tumor tissue were reported in some canine cases of HS. SHP2 consists of two src homology 2 domains (N-SH2 and C-SH2), a protein tyrosine phosphatase (PTP) domain, and a C-terminal tail with tyrosyl phosphorylation sites. In the absence of signal, SHP2 exists in a folded auto-inhibitory conformation with the N-SH2 domain docked into the catalytic cleft of the PTP domain. Recruitment of SHP2 to an activated RTK induces conformational change in SHP2 from folded to the open-active state by dissociation of the N-SH2 domain from the PTP domain. Glu76 in the

human N-SH2 domain is located within the region of the N-SH2/PTP interface and is known as a hotspot of driver mutations (e.g., p.Glu76Lys) in various tumors. Mutations in the region of the N-SH2/PTP interface have been shown to shift the conformational equilibrium toward the open state, resulting in constitutively activation of SHP2 and aberrant cellular proliferation. A similar conformational change in SHP2 may activate downstream signaling molecules and subsequent neoplastic proliferation of histiocytes in canine HS harboring SHP2 mutations.

SHP099 is a recently developed, highly selective, orally bioavailable, potent allosteric inhibitor of SHP2. The compound stabilizes SHP2 in an auto-inhibited conformation by concurrent binding to the interface of the N-SH2, C-SH2, and PTP domains. SHP099 has been shown to have anti-proliferative activity in human tumor cell lines by inhibition of wild-type SHP2 that is activated by oncogenic driver RTKs such as EGFR, FLT-3, and KIT. More recently, SHP099 has been demonstrated to inhibit the growth of a human leukemia cell line expressing mutant SHP2. Although there is no evidence that canine HS harbors oncogenic driver RTKs, the finding of Sun et al. in 2018 suggests the potential utility of SHP099 for growth inhibition of canine HS cells harboring mutant SHP2 proteins.

Although some canine cases of HS are considered to carry SHP2 mutations that

locate within the activation regulatory regions of SHP2 in tumor cells, the precise mutational positions in SHP2 were not clear because entire coding nucleotide sequence of canine *PTPN11* has not been determined. In addition, functional role of the SHP2 mutations on structure and activity of SHP2 and growth of HS cells is unclear. Therefore, uncovering the effects/roles of SHP2 mutations in the SHP2 activation and growth of HS cells is indispensable to open the way for establishment of SHP2-targeted new therapy with SHP099.

In the current study, to make a foundation of SHP2-targeted therapy for canine HS, entire nucleotide coding sequence of canine *PTPN11* was identified and expression and mutation status of SHP2 in canine HS cell lines were examined. Moreover, effects of mutations on the structures and the phosphatase activities of canine SHP2 were examined. Furthermore, the growth inhibitory properties of SHP099 for HS cells were investigated *in vitro* and *in vivo*.

1. Expression levels and mutation status of *PTPN11*/SHP2 in HS cell lines.

To investigate expression levels and mutation status of *PTPN11*/SHP2 in HS cell lines, firstly, entire coding nucleotide sequence of canine *PTPN11* was determined using cardiac cDNA isolated from healthy dog (registered with NCBI: GenBank

accession number, MK_372881.1). Subsequently, expression levels and mutation status of *PTPN11*/SHP2 in HS cell lines were examined. All of six HS cell lines examined with western blot analysis were expressed SHP2 and four out of nine HS cell lines had mutations in *PTPN11*/SHP2 (p.Ala72Gly, CHS-1; p.Glu76Gln, CHS-3; p.Glu76Ala, CHS-6; p.Gly503Val, ROMA). These mutations were located at within the region of the N-SH2/PTP interface that plays a key role to maintain SHP2 as the closed conformation. Therefore, mutations of *PTPN11*/SHP2 identified in HS cell lines are considered to play a pivotal role for their growth via alteration of SHP2 function.

2. Analysis of activation status and underlining structural changes of canine SHP2 harboring a mutation

Using recombinant protein and *in silico* approach, activation status and underlining structural changes of canine SHP2 harboring a mutation were investigated. Recombinant canine SHP2 harboring p.Ala72Gly, p.Glu76Gln and p.Glu76Ala showed constitutive phosphatase activities, while phosphatase activity was not detectable in wild-type SHP2 and SHP2 harboring a p.Gly503Val mutation. The activities SHP2 harboring p.Ala72Gly, p.Glu76Gln and p.Glu76Ala were inhibited by SHP099. *In silico* analysis suggested that mutations p.Glu76Gln and p.Glu76Ala but not p.Ala72Gly and

p.Gly503Val promote shifting of the SHP2 conformation from folded to open-active state. These findings suggest that not all mutations cause constitutive activation of SHP2 and the impact on activity and structure of SHP2 may vary depending on the location and/or type of the mutation. Specifically, SHP2 Ala72/Glu76 mutations are considered potential therapeutic targets for HS and SHP099 might be a therapeutic agent for HS harboring these mutations.

3. Effect of SHP099 on growth of HS cells.

To examine if SHP099 has toxic effect on HS cells, growth inhibitory activity of SHP099 against HS cell lines was evaluated *in vitro* and *in vivo*. Among six HS cell lines, SHP099 potently suppressed the growth of CHS-3 (p.Glu76Gln) and CHS-6 (p.Glu76Ala) cells. In contrast, other cell lines harboring SHP2 p.Ala72Gly, p,Gly503Val or wild-type had lower susceptibilities to SHP099. In HS xenograft mouse model using CHS-6 (p. Glu76Ala), SHP099 exhibited potent anti-tumor activity. These findings suggest that SHP2 harboring p.Glu76Ala and p.Glu76Gln mutations are the therapeutic targets for HS and SHP099 is considered an attractive agent for treatment of HS carrying these mutations.

In conclusion, p.Glu76Gln and p.Glu76Ala are activating mutations of SHP2 and play a pivotal role for survival/growth of HS cells carrying these mutations. Targeting p.Glu76Gln and p.Glu76Ala SHP2 with SHP099 may be a new therapeutic strategy for canine HS.