Studies on growth inhibitory effects of dasatinib against canine histiocytic sarcoma cell lines

Summary

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Canine histiocytic sarcoma is an aggressive, often lethal tumor that arises from the histiocytic lineage including macrophages and dendritic cells. There is a highly elevated incidence in several breed, including Bernese Mountain Dog, Flat Coated Retriever, Rottweiler and Golden Retriever, especially, Bernese Mountain Dog and Flat Coated Retriever may share genetic characteristics contributing to tumor initiation and progression.

For the treatment of HS, Chemotherapy is often used alone or in combination with radiation in an adjuvant setting or for locally advanced or metastatic disease. Altough overall response rate of HS cases with gross lesions treated by lomustine (CCNU) was around 46%, median survival time of these cases was short with a range of 3 to 6 month. Therefore, a new therapeutic approach is required for the treatment of HS.

The survival and growth of tumor cells are usually associated with multiple abnormalities, including dysregulation of growth signaling pathway, cell cycle, apoptosis, angiogenesis, DNA replication/repair, etc. Therefore, it has been believed to be difficult to elicit tumor cell death by targeting only one aberrant molecular mechanism. However, recently, it has been shown that certain tumor cells strongly depend on one or a few these abnormalities. In these tumors, chemical compounds have been reported to elicit tumor cell death by targeting aberrant mechanism. Compounds for inhibiting these abnormal molecular selectively are named ‘molecular target drug’. Major type of molecular target drug is a protein kinase inhibitor. These inhibitors show anti-tumor

effects through binding to ATP binding site of activated kinases. In the past, marked efficacy of molecular target drugs has been reported in human chronic myelogenous leukemia with Bcr-Abl gene, human non-small lung cancer with EGFR mutation, human advanced non-small-cell lung cancer harboring ALK gene rearrangement, and canine and feline mast cell tumor with KIT mutation.

From here, it is considered to explore the possibility of new therapeutic approach using molecular target drug to canine HS with treated incompletely. It is necessary to detect a specific molecular mechanism which strongly regulates the survival and growth of HS in order to establish a molecular target therapy against HS. In the past, some aberrant molecular mechanisms such as abnormal expression of tumor-suppresser gene or abnormality of transcriptional regulator have been reported in HS. However, molecular target therapy against canine HS has not been established because specific molecular mechanism which strongly regulates the survival and growth of this tumor has not been detected.

In this study, to establish molecular target therapy against canine HS, we first identified specific molecular mechanism which strongly regulates the survival and growth of HS cells using chemical library, including many types of molecular inhibitors. Secondly, we evaluated genetic abnormality and activity of downstream signaling of targeted molecular of candidate compound, and searched for other targeting molecular of this compound. Lastly, we evaluated *in vivo* anti-tumor effect of the compound which

inhibited the growth of HS cells *in vitro*, using HS cell xenografted mouse models.

1. A global search to identify crucial molecular mechanism in the survival and proliferation of HS cell lines

For the growth inhibitory assay of the compounds that have growth inhibitory effect in HS cells, two HS cell lines (CHS-1 and MHT-2) and 219 compounds were used. Dasatinib clearly inhibited the growth of CHS-1 cells. We then focused on dasatinib and examined its growth inhibitory property against six HS cell lines. Dasatinib clearly inhibited the growth of four HS lines (CHS-1, CHS-2, CHS-4 and CHS-7) with calculated IC50 values of 5.4 to 54.5 nM concentrations. Among the 219 compounds, 15 targets that overlap with those of dasatinib. These compounds target Bcr-Abl, Src family kinase, Kit or PDGFR. They showed no growth inhibition in dasatinib-sensitive CHS-1 cells. Therefore, it is apparent that dasatinib does not act through the suppression of these recognized molecular targets and the inhibition of another target such as EphA2 or undefined targets may explain the effect seen on HS cells.

In this study the growth of some subsets of HS cells seems to be critically dependent on a specific kinase targeted by dasatinib and this on-target activity of dasatinib most likely resulted in marked growth inhibition in these HS cells.

2. Analysis of targeted molecular of dasatinib in HS cells

To find out the mechanism of growth inhibitory by dastinib in HS cells, we evaluated expression level, mutation and gene amplification level of EphA2, Bcr-Abl, Src family kinase, Kit, and PDGFR genes and activity level of downstream signaling pathways of these molecular using dasatinib sensitive cell lines (CHS-1, CHS-2, CHS-4, and CHS-7) and dasatinib insensitive cell lines (MHT-2 and CHS-5). In addition, we clarified new target molecular of dasatinib in CHS-1 cells with global analysis of phosphorylated protein.

Gene expression and genomic amplification of EphA2 and Bcr were not observed in dasatinib sensitive cell lines. Mutation of EphA2, Abl, Bcr, Src, and Yes was not existed in CHS-1 cells. Moreover, phosphorylation of AKT, ERK1/2, and STAT3 as downstream signaling molecular was not observed in dasatinib sensitive HS cell lines. These results suggested the growth inhibitory effect of dasatinib to HS cells may be mediated by not inhibition of well-known target molecular but inhibition of new undetected molecular. As the result of phosphorylated protein analysis in CHS-1 cells, it was shown that constant phosphorylation of 14-3-3 protein gamma in CHS-1 cells and this phosphorylation was inhibited by dasatinib. It seemed that proliferation of CHS-1 cells is strongly regulated by constant phosphorylation of 14-3-3 protein gamma because phosphorylated 14-3-3 protein gamma promotes cell cycle with effecting to ATR-Chk1-Cdc25A pathway related to DNA damage checkpoint mechanism. However,

14-3-3 protein gamma has no kinase activities. Therefore, it suggested that growth inhibitory effect of dasatinib was mediated by directly inhibition some kind of kinase in the JNK pathway which is upstream pathway of 14-3-3 protein gamma.

3. Effect of dasatinib in canine HS xenografted mouse moldel

The in vivo growth inhibitory activity of dasatinib against CHS-1 cells was evaluated by using xenograft mouse model. Dasatinib clearly suppressed the growth of CHS-1 xenografted tumors. Tumors from the dasatinib-treated mice showed a significant decrease in mitotic index and Ki-67 index compared to controls. On the other hand, the apoptotic index was significantly increased in tumors excised from the dasatinib-treated mice when compared to controls. These results suggest that the inhibition of tumor growth in CHS-1 cells may be attributed to the inhibition of cell division and the increased cell death by dasatinib.

The inhibition of cell division may be caused by inhibition of phosphorylation of 14-3-3 protein gamma by dasatinib in the tumors because it was speculated that dasatinib inhibits cell cycle progression by inhibiting constant phosphorylation of 14-3-3 protein gamma in CHS-1 cells in vitro. In contrast, the mechanism of induction of apoptosis is not identified.

In this study, dasatinib has growth inhibitory effect *in vivo*. Therefore, dasatinib will be expected to use selected clinical HS case with phosphorylation of 14-3-3 protein gamma

in the tumor.

In conclusion, our study suggested that a constant phosphorylation of 14-3-3 protein gamma plays a crucial role in the growth of some specific type of HS cells. Dasatinib showed growth inhibitory effects in HS cells with prolonged phosphorylation of 14-3-3 protein gamma both *in vitro* and *in vivo.* Therefore, dasatinib has potent efficacy for the treatment in the selected clinical HS cases with constant phosphorylation of 14-3-3 protein gamma in these tumor cells.