Studies on *KIT* mutations and toceranib susceptibility in canine mast cell tumor

Summary

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In the treatment of canine mast cell tumor (MCT), a kinase inhibitor toceranib is frequently used and has been demonstrated anti-tumor activity in certain dogs. Toceranib is a multi-kinase inhibitor that targets receptor tyrosine kinase such as KIT, PDGFR, and VEGFR. One of the mechanisms underlying the action of toceranib is inactivation of KIT that is constitutively activated by a mutation in MCT cells. However, the therapeutic activity of toceranib does not necessarily correlate with the presence of the KIT mutation and thus individualized therapy with toceranib have not been established in canine MCTs. The major reason of this issue is considered that only hotspot of mutation in KIT (internal tandem duplication [ITD] mutation in KIT exon 11) has been evaluated for analysis of the correlation between response to toceranib and KIT mutation status. Although some mutations other than ITD mutation in exon 11 have been reported in canine MCTs, comprehensive mutation analysis of KIT in canine MCT has not been done and thus overall mutation status of KIT in canine MCT remains unclear. Moreover, properties of each mutant KIT other than KIT harboring ITD mutation in exon 11 are poorly understood. Therefore, to establish individualized therapy with toceranib, it is necessary to understand detailed variation of KIT mutation and functional effects of each mutations on KIT.
To make a foundation of individualized therapy with toceranib in canine MCT, following studies were performed: Chapter 2, comprehensive mutation analysis of KIT was performed on genomic DNA samples of 164 dog MCTs using next-generation sequencing (NGS) approach. Moreover, recombinant KIT proteins containing mutations identified by NGS and reported but not characterized mutations were prepared and characterized; Chapter 3, because some mutant KIT that confer resistance to toceranib were identified in Chapter 2, this chapter was focused on toceranib-resistant KIT mutations. In this context, generation of KIT mutations in MCT cell lines were investigated using NGS during the process of acquisition of resistance to toceranib; Chapter 4, to develop a strategy to overcome toceranib-resistance in MCT, effects of SHP2 inhibition on the growth of toceranib-resistant MCT cell lines was examined.

1. Identification of KIT gene mutations in canine MCT cases and analysis of phosphorylation status of mutant KIT

To comprehensively examine KIT mutations in canine MCTs, NGS analysis of all exons of KIT was performed using genomic DNA in 164 canine MCT tumor samples. From this analysis, 35 mutations that have not been previously reported were identified; specifically, 12 in the immunoglobulin-like region, 20 in the juxtamembrane region, two in the kinase region and
one in the C-terminal region. In addition, recombinant KIT proteins harboring mutations including mutations that found with NGS analysis and that reported but not characterized were prepared and the phosphorylation level and toceranib susceptibility were analyzed. The mutations have different characteristics in terms of phosphorylation levels and degree of toceranib susceptibility. Moreover, low frequency but some mutations conferred toceranib resistance to KIT. Thus, it was considered that some MCT cases have minor clones that have a predisposition of toceranib resistance in tumor tissue before starting the toceranib treatment. These findings suggest that it is important to take characteristics of each mutation into consideration for development of individualized therapy using toceranib.

2. Generation of toceranib-resistant canine MCT cell lines and analysis of KIT mutation during clonal expansion and subsequent acquisition of resistance to toceranib

Comprehensive KIT mutation analysis revealed the presence of mutations that could confer toceranib resistance to MCT cells. These mutations are likely to make toceranib treatment difficult and thus are critical for development of individualized toceranib therapy. Therefore, to clarify the developmental process of toceranib-resistant KIT mutation, toceranib-resistant cell lines were generated from cloned MCT cell lines and performed NGS analysis of their KIT genes.
The toceranib-resistant cell lines but not parental toceranib-sensitive cell line had multiple secondary mutations (c.2037T>A, p.(Asn679Lys); c.2456A>T, p.(Asp819Val); c.2456A>G, p.(Asp819Gly)) in addition to the primary mutation(c.1523A>T, p.(Asp508Ile)). The recombinant KIT protein harboring these mutations showed toceranib resistance. Therefore, it was considered that the secondary mutation of KIT plays an important role on resistance to toceranib in canine MCTs. In addition, considering the results in Chapter 2, MCT cells carrying a toceranib-resistant mutations in KIT may occur via two different process, in which one is selective expansion of pre-existing minor clone carrying such mutation in KIT under the treatment of toceranib and the other is de novo emergence by continuous exposure to toceranib.

3. **Analysis of the effect of SHP2 inhibitor on toceranib-resistance canine MCT cell lines**

The results of Chapters 2 and 3 indicate that KIT harboring a toceranib resistance mutation plays important role on acquiring toceranib resistance of canine MCTs. Therefore, in Chapter 4, development of a strategy to overcome toceranib-resistance of MCTs was attempted. For this purpose, protein-tyrosine phosphatase SHP2, which regulates downstream signals of KIT, was targeted with SHP2 inhibitor SHP099. Although SHP099 alone did not show obvious growth inhibitory effect to toceranib-resistant MCT cell lines, it showed potent growth inhibitory effect
when combined with toceranib. From these findings, SHP099 was considered to be a potential therapeutic approach for both MCTs that have already acquired toceranib resistance as well as that are toceranib-naïve but have minor toceranib-resistant clones in tumor tissue.

In conclusion, there are various types/characteristics of mutations in KIT in canine MCTs. Therefore, it is necessary to consider the difference of characteristics among each mutation type for development of individualized therapy with toceranib in canine MCTs. Particularly, it should be noted that there are tumor cells carrying toceranib-resistant KIT mutations that may pre-exist in toceranib-naïve tumor tissue or may occur de novo in tumor tissue during toceranib treatment. For canine MCTs carrying toceranib-resistant clones, a combination therapy of toceranib with SHP099 might be a potential therapeutic approach.