Studies on the development of resistance to imatinib in canine mast cell tumor

（犬の肥満細胞腫におけるイマチニブ耐性化に関する研究）

Summary

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Canine mast cell tumor (MCT) is one of the common skin tumors and accounts for 20 % of all canine cutaneous tumors. Chemotherapy, using vinblastine and/or lomustine is sometimes needed in the treatment of MCT, especially in the cases of advanced clinical stage and/or high grad of tumor. Recently, in addition of these chemotherapeutic agents, KIT-targeted kinase inhibitor imatinib came to be used for the treatment of MCT in dogs.

KIT is a type III receptor tyrosine kinase encoded by *KIT*. By binding with its ligand stem cell factor (SCF), it is phosphorylated and activates downstream signal transduction, leading proliferation, migration, maturation and survival of cells. Approximately 26 % of canine MCT have *KIT* mutations. These mutations cause a constitutive auto-phosphorylation of KIT, resulting in the neoplastic growth of mast cells.

Imatinib binds to the ATP-binding site within KIT and suppresses phosphorylation of this receptor. Therefore, imatinib shows therapeutic activity in canine cases of MCT with *KIT* mutation. However, they eventually develop resistance to imatinib in the course of treatment. Although this is a crucial issue in the treatment of MCT in dogs, the molecular mechanisms of acquiring of resistance to imatinib have not been clarified.

The purpose of this study is to clarify the molecular mechanisms of imatinib resistance in canine MCT. Firstly, nucleotide sequences of *KIT* were analyzed using tumors collected from the MCT cases that acquired resistance to imatinib. Secondly, imatinib resistant MCT sub-lines were established from the imatinib sensitive MCT cell lines and characterized their biological changes. Finally, using these sub-lines,

molecular mechanisms of imatinib resistance were investigated.

1. Analysis of nucleotide sequences of *KIT* using tumors collected from the cases of MCT that acquired resistance to imatinib

Nucleotide sequences of *KIT* were analyzed using tumors collected from six cases of MCT that acquired resistance to imatinib. All dogs had a primary mutation in *KIT*. A second mutation (c.2463T>A, p.Asp815His) in *KIT* was found in a tumor in one dog. In contrast, other five cases had no second mutation in *KIT*. A same substitution mutation at the corresponding residue (p.Asp816His) has been found in imatinib resistant human GIST. Moreover, phosphorylation of the human mutant KIT (p.Asp816His) has been shown not to be suppressed by imatinib. Therefore, the second mutation (c.2463T>A, p.Asp815His) could be associated with imatinib resistance in this dog. In the five dogs that did not have second mutation in *KIT*, it could be possible that molecular mechanisms other than second mutation in *KIT* play a crucial role in the acquiring of resistance to imatinib.

2. Establishment and characterization of imatinib resistant mast cell tumor sub-lines

Using imatinib-sensitive canine MCT cell lines CoMS (IC50; 0.04 μM) and VI-MC (IC50; 0.27 μM), imatinib resistant sub-lines rCoMS1 (IC50; 9.0 μM, from CoMS), rVI-MC1 (IC50; 1.86 μM, from VI-MC), and rVI-MC10 (IC50; 12.2 μM, from VI-MC) were established by culturing in increasing concentrations of imatinib.

In rCoMS1, overexpression of KIT and its phosphorylation was observed. Phosphorylation of the overexpressed KIT was not suppressed by imatinib, suggesting association of overexpression of KIT and imatinib resistance. Both in rVI-MC1 and rVI-MC10, a second mutation of *KIT* (c.2443G>C) that located in exon 17 was identified. The phosphorylation of KIT was not suppressed by 1 μM but suppressed by 10 μM of imatinib in both rVI-MC1 and rVI-MC10. From these findings, it was suggested that the second mutation in *KIT* contributed to the resistance to 1 μM of imatinib. However, it was considered that other molecular mechanisms underlie the imatinib resistance in rVIMC10.

3. Molecular mechanisms of acquiring resistance to imatinib in CoMS

Association between overexpression of KIT and imatinib resistance was investigated using rCoMS1. From the protein synthesis inhibiting study, it was shown that increase of KIT expression on rCoMS1 was caused by reduced turnover of KIT with prolonged half-life. Moreover, this decrease of KIT turnover was caused by decrease of ubiquitination of KIT triggered by imatinib.

KIT overexpressed on rCoMS1was decreased by culturing in the absence of imatinib. This down-regulated KIT was re-upregulated by re-culturing in the presence of imatinib. In consistent with KIT expression status, rCoMS1 represented changes of imatinib sensitivity; namely, rCoMS1 with decreased expression of KIT was sensitive to imatinib but that re-upregulated expression of KIT was insensitive to imatinib.

From these findings, overexpression of KIT on rCoMS1 was considered to be

caused by retardation of KIT degradation via inhibition KIT ubiquitination by imatinib and to play a crucial role in resistance to imatinib in this cell line.

4. Molecular mechanisms of acquiring resistance to imatinib in VI-MC

The effects of the second mutation (c.2443G>C) in *KIT* on the phosphorylation status of KIT and its sensitivity against imatinib were examined using recombinant mutant KIT expressed 293 cells. Regardless of the presence or absence of primary mutation, the mutant KIT harboring the second mutation showed ligand-independent phosphorylation that was not suppressed by imatinib. This indicates that the second mutation in *KIT* is responsible for the imatinib resistance in rVI-MC1 and rVI-MC10.

Despite of carrying a same second mutation, tolerability against imatinib was different between rVI-MC1 and rVI-MC10. Therefore, phosphorylation status of KIT downstream signaling proteins ERK, AKT and STAT3 was then examined. ERK was constitutively phosphorylated in both cell lines. This phosphorylation was suppressed by 10 μM of imatinib in rVI-MC1 but not in rVI-MC10. ERK phosphorylation in rVI-MC10 was also not suppressed by KIT/SFK inhibitor. KIT/SFK-independent activation of ERK would be involved in imatinib resistance in rVI-MC10.

In summary, the frequency of second mutation in *KIT* could be low in imatinib resistant canine MCT. Overexpression of KIT is one of a cause of acquiring resistance to imatinib in the cases without the second mutation in *KIT*. In the cases that have a second mutation in *KIT*, the second mutation was suggested to be a cause of the

resistance to imatinib. Moreover, KIT/SFK-independent activation of ERK would be involved in imatinib resistance when the neoplastic cells are exposed to higher concentrations of imatinib.