Studies on growth mechanisms and their suppression in canine squamous cell carcinoma cells

Summary

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Squamous cell carcinoma (SCC) is a common canine tumor that arises from the squamous epithelium in a variety of locations. Due to its locally invasive nature, local therapies such as surgery and surgery combined with radiotherapy are primarily used for the treatment. In some cases in which local therapy is not applicable or has not been successful, chemotherapy is the treatment option; however, canine SCC is usually chemotherapy resistance. Therefore, development of new therapeutic strategies is needed for such cases. For that purpose, it is indispensable to identify targetable molecular mechanism critically involved in survival/growth of canine SCC cells. Because previous studies have been demonstrated that survivin and EGFR are overexpressed in canine SCC tissues, these molecules are considered to be closely associated with growth of canine SCC and thus are attractive new therapeutic targets.

Survivin, a member of the IAP family, functions as a promoter of cell division and an inhibitor of apoptosis. Survivin is highly expressed in many human malignancies but rarely expressed in normal tissues except for bone marrow and genital organs. Recently, a potent survivin inhibitor YM155 has shown some efficacies for human patients with various tumors in phase I and II clinical trials. Therefore, inhibition of survivin using YM155 may be a new therapeutic strategy in canine SCC.

EGFR, a receptor tyrosine kinase, is widely expressed in epithelial cells and

has been shown to play a crucial role in cell growth. Overexpression of EGFR has been observed in many human SCCs and has been demonstrated to play a crucial role in tumor growth. Additionally, it is rare but some SCCs have gain-of-function mutations in EGFR that constitutively phosphorylate EGFR and promote tumor growth. Currently, although no therapeutic approach targeting EGFR has been established in human SCC, human patients with lung adenocarcinoma carrying EGFR mutation has been successfully treated with EGFR inhibitors such as afatinib and osimertinib. Therefore, similar to targeting survivin with YM155, targeting EGFR by atatinib or osimertinib is potential therapeutic strategy for canine SCC.

Most of molecular targeted drugs including YM155 and EGFR inhibitors are developed for human tumors, while that developed for dogs are only masitinib and toceranib. Toceranib is a multikinase inhibitor and has been shown to anti-tumor effect for various canine tumors. Considering that toceranib is multikinase inhibitor and is one of the few molecular targeted drugs for dogs, it is meaningful to clarify whether toceranib has a growth inhibitory effect on canine SCC cells and to identify the target molecule if it has a growth inhibitory effect to SCC.

In this study, to establish new therapeutic approaches for canine SCC using molecular target drugs, sensitivities of canine SCC cell lines to YM155, afatinib,

osimertinib, and toceranib were examined. Moreover, by focusing to YM155 and afatinib, growth inhibitory mechanisms in canine SCC cell lines were investigated. Furthermore, anti-tumor effect of afatinib was tested using a canine SCC cell line *in vivo*.

1. Analysis of sensitivities to survivin, EGFR, and multi-tyrosine kinase inhibitors in canine SCC cell lines

To examine if survivin and EGFR are therapeutic targets in canine SCC cells, sensitivities of seven canine SCC cell lines to YM155, afatinib and osimertinib were examined. Additionally, the same assay was also performed using toceranib. YM155 and afatinib potently and selectively inhibited growth of HAPPY and SQ4 cells and POCO and CSCC-R1 cells, respectively. In contrast, osimertinib and toceranib did not show such growth inhibitory effects against canine SCC cell lines. These results suggested that survivin or molecule(s) targeted by afatinib may be crucial role in the growth of certain canine SCC cells, rendering these molecules are attractive as therapeutic targets for canine SCC.

2. Analysis of mechanism of action of YM155 in canine SCC cell lines

To clarify the mechanism of action of YM155 in canine SCC cell lines, expression levels of survivin in seven canine SCC cell lines were examined. Moreover,

changes of survivin expression levels and status of cell death pathways were examined in HAPPY and SQ4 cells under the treatment with YM155. Both YM155-sensitive HAPPY and SQ4 cells highly expressed survivin that was suppressed by YM155. The suppression mechanisms of survivin by YM155 were differed between HAPPY and SQ4 cells, in which YM155 inhibited survivin expression by suppression of *survivin* transcription in HAPPY cells, while it inhibited survivin expression via post-translational mechanism in SQ4 cells. Moreover, there were differences in YM155-induced cell death pathways between these cell lines. Although YM155 induced autophagy and subsequent PARP-dependent apoptosis in both cell lines, HAPPY cells primarily underwent cell death via PARP-dependent apoptosis, while there were two different cell death mechanisms including PARP-dependent apoptosis and probably autophagic cell death in SQ4 cells. From these findings, targeting survivin with YM155 may be a potential new therapeutic approach for canine SCC with high expression of survivin.

3. Investigation of the target molecule of afatinib in canine SCC cell lines

To identify the target molecule(s) in afatinib-sensitive cell lines, expression and phosphorylation status of ErbB family proteins, nucleotide sequences of known afatinib target molecules, and phosphorylation status of intracellular signaling molecules were analyzed using canine SCC cell lines. In addition, a comprehensive

analysis of phosphorylated proteins was performed using POCO cells. From the protein and gene analysis of known afatinib target molecules, no actual target in afatinib-sensitive cells was identified. In a comprehensive analysis of phosphorylated proteins using POCO cells, it was found that afatinib mainly suppressed the activation of the MAPK pathway. These findings suggest that the MAPK pathway plays a crucial role in cell proliferation in certain canine SCCs and the suppression of the MAPK pathway using afatinib may be a new therapeutic approach in such SCCs.

4. Anti-tumor activity of afatinib in canine SCC xenograft mouse model

In vivo anti-tumor activity of afatinib against canine SCC was investigated using POCO cells xenograft mice. For comparison, osimertinib, another EGFR inhibitor, was also used in this study. Afatinib showed a remarkable anti-tumor effect against POCO cells. In contrast, osimertinib showed almost no anti-tumor effect. These results suggest that afatinib has anti-tumor activity against certain SCC cells *in vivo*. Considering that afatinib suppressed the MAPK pathway in POCO cells in chapter 4, afatinib would be worth proceeding to a clinical trial in SCC cases in which tumoral MAPK pathway is activated.

In conclusion, it was suggested that the expression of survivin or phosphorylation of the MAPK pathway plays a crucial role in growth/survival of

certain canine SCCs. YM155 and afatinib may be promising as new therapeutic strategies for such canine SCCs.