論　文　内　容　要　旨

The transition of tissue inhibitor of metalloproteinases from TIMP-4 to TIMP-1 induces aggressive behavior and poor patient survival in dedifferentiated liposarcoma via YAP/TAZ activation

(TIMP-4 から TIMP-1 への発現移行は YAP/TAZ 活性化を介して脱分化脂肪肉腫における侵襲性や低生存率を誘導する)

Carcinogenesis, 2019, in press.

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Liposarcoma (LS) is the most common soft-tissue malignancy constituting 15-20% of all soft-tissue tumors. Well-differentiated liposarcoma (WDLS) closely resembles mature fat cells; while de-differentiated liposarcoma (DDLS) consist of a WDLS component with areas of dedifferentiation of non-lipogenic component. DDLS is aggressive and has an overall poor prognosis and survival than WDLS. Current therapeutic options are less effective for locally aggressive or metastatic DDLS, hence novel therapeutic strategies are of utmost need. The Hippo pathway consists of a series of kinases such as mammalian STE20-like protein kinases 1 and 2 (MST1/2), and large-tumor-suppressor-homologue 1 and 2 (LATS1/2), which form a complex with SAV1 adaptor proteins and MOB1A/B. Yes-associated protein and its homologue transcriptional coactivator with PDZ-binding motif (YAP/TAZ) are the effector of Hippo signaling pathway and work as a co-transcription factor with various transcription factors, mainly TEAD (TEA domain family member). LATS1/2 kinases upon activation phosphorylate YAP/TAZ, causing their cytoplasmic localization and degradation through the ubiquitin-mediated proteasomal degradation. YAP/TAZ have been established as oncoproteins constitutively activated to cause aberrant cell proliferation, migration and apoptosis through their target genes (such as CTGF, CYR61) in various types of malignancies including DDLS. Therefore, understanding the molecular mechanism regulating YAP/TAZ activation can warrant advanced therapeutic approaches for LS. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of metalloproteinases and comprise of four members,

TIMP-1 to -4. TIMP-1 shows high expression that correlates with poor prognosis in breast, pancreas, gastric, colon, brain, prostate and other cancers. Recently, we have elucidated a novel biological mechanism that TIMP-1 after binding with CD63 activates YAP/TAZ to increase proliferation in various cancer cell lines like cervical, breast and oral cancer. I hypothesize if TIMP-1 can be a key molecule to explain the aberrant YAP/TAZ activation in DDLS. TIMP-4 has been reported to share the same CD63 receptor as TIMP-1 and is found in high levels in normal fat tissues. As WDLS show a histology of mature fat cells, I speculated that TIMP-4 expression might be high in WDLS as compared to DDLS, and its function might be opposite to that of TIMP-1.

Thus, my aim is to clarify the expression and function of TIMP-1 and -4 through YAP/TAZ regulation in WDLS and DDLS.

Part I: TIMP-1 and TIMP-4 are upregulated in DDLS and in WDLS respectively

I explored databases for TIMP-1/TIMP-4 expression and relationship with patient survival and prognosis along with expression pattern in cell lines.

GSE30929 (Prognoscan) and Gobble Sarcoma (Oncomine) database analysis showed high expression of TIMP-1 in patients with low distant metastasis free survival, whereas TIMP-4 expression is higher in patients with increased distant recurrence free survival. Databases also showed high

expression of TIMP-1 and low expression of TIMP-4 in DDLS, while high expression of TIMP-4 and low expression of TIMP-1 in WDLS. Consistent with database analysis, TIMP-1 was high in SW872 and FU-DDLS (DDLS) cells, while TIMP-4 was high in 94T778 (WDLS) cells. YAP/TAZ levels were higher in SW872 and FU-DDLS cells than in the 94T778 cells.

Part II: TIMP-1 promotes proliferation and migration via YAP/TAZ activation

This was conducted to know the role of TIMP-1 in LS through YAP/TAZ signaling. I established TIMP-1 knockdown (shTIMP-1) and control group (shscramble) stable clones using SW872 and FU-DDLS cell lines. Using 94T778 cell lines, TIMP-1 over-expressing stable clones and empty vector control were established. Experiments such as proliferation assay, migration assay, apoptosis assay, colony formation assay and expression of target genes with RT-PCR were performed. Rescue experiments using YAP5SA (constitutively active YAP) vector was conducted AZ. Expressions of TIMP-1-YAP/TAZ signaling molecules were analyzed by Western blot and RT-PCR.

TIMP-1 knockdown reduced YAP/TAZ levels and increased LATS1 with its phosphorylation at threonine 1079. TIMP-1-knockdown suppressed YAP/TAZ levels were rescued back by MG132, a proteasome inhibitor. Moreover, TIMP-1 knockdown decreased nuclear translocation of YAP/TAZ. Activated LATS1/2 phosphorylate YAP/TAZ to induce their cytoplasmic retention and

degradation through ubiquitin-proteasome pathway. TIMP-1 knockdown suppressed target gene *CTGF* expression, cell growth, migration and increased apoptosis in DDLS cells. YAP5SA could rescue them back. Similar results of YAP/TAZ activation leading to increased cell growth, migration and decreased apoptosis was seen with TIMP-1 overexpressing 94T778 cells which was rescued by Verteporfin (inhibitor of YAP/TAZ).

Part III: TIMP-4 inhibits cell proliferation and migration through YAP/TAZ inactivation in WDLS cells

My next question was whether TIMP-4 has opposite roles to that of TIMP-1 or not. Using 94T778 cell lines, I established stably TIMP-4 knockdown (shTIMP-4) and shscramble cells and performed proliferation assay, migration assay, apoptosis assay, expression of target genes with RT-PCR and rescue experiments using siRNA knockdown of YAP/TAZ as well as Verteporfin

TIMP-4 knockdown 94T778 cells showed upregulation of YAP/TAZ than the shscramble cells. TIMP-4 knockdown promoted YAP/TAZ target genes *CYR61* expression along with cell proliferation, migration and decreased apoptosis than the shscramble group. Both, Verteporfin treatment and si-YAP/TAZ knockdown could suppress these.

Part IV: Recombinant TIMP-4 reduces oncogenic potential of DDLS via YAP/TAZ inactivation

Finally, to know the therapeutic role of TIMP-4 in DDLS, I treated SW872 and FU-DDLS with variable concentrations of recombinant TIMP-4 (rTIMP-4) which induced phosphorylation of YAP at S127 and suppressed proliferation and migration of SW872 and FU-DDLS cells. Moreover, target genes expression of SW872 cells was also suppressed.

Part V: Expression pattern of TIMP-1 and -4 in LS patient samples

Patient samples of WDLS and DDLS tissues sections were analyzed for immunohistochemical expression patterns of TIMP-1 and TIMP-4 in LS. Immunohistochemistry showed that tissue expression of TIMP-1 was significantly high in DDLS but low expression in WDLS. Similarly, TIMP-4 was high in WDLS but low in DDLS.

Conclusion:

Switching of TIMP-4 to TIMP-1 expression during transition from a WDLS to a DDLS phenotype leading to aberrant YAP/TAZ signaling is an interesting novel phenomenon observed in LS. Tumor tissue expression of TIMP-1 or TIMP-4 could be possibly used as a prognostic marker for LS. Identification of this novel TIMP-1/-4-YAP/TAZ signaling axis is the first step to understand the underlying pathogenetic mechanism in LS. This may also warrant future possibilities of targeting key molecules in development of therapeutic novelties in treating LS.