博 士 論 文（ 要 約 ）

Down regulation of TGFβ pathway in leukemogenesis by *SETBP1* mutation in *ASXL1*-mutated MDS

（*SETBP1* と *ASXL1* 遺伝子変異を起因とする白血病における TGFβ シグナル経路の発現低下の解析）

齋 賀　真 言

論文の内容の要旨

論文題名　Down regulation of TGFβ pathway in leukemogenesis by *SETBP1* mutation in *ASXL1*-mutated MDS

（*SETBP1* と *ASXL1* 遺伝子変異を起因とする白血病における TGFβ シグナル経路の発現低下の解析）

氏名　　齋賀 真言

Myelodysplastic syndromes (MDS) are heterogeneous group of clonal hematological disorders commonly found in the elderly population. They are characterized by one or more peripheral cytopenias, however inside bone marrow is generally hypercellular. Malignant cells display aberrant morphology and blocked differentiation, resulting in ineffective hematopoiesis. Their risks are categorized into 4 or 5 ranks along with scoring systems established by various groups, predicting the outcome and the rate of leukemic transformation. The mean survival time at diagnosis depends on the risk category, varying from several months to 10 years around, and these outcomes are never easy for patients. Besides, leukemic transformation of MDS has been the biggest impact on the mortality of MDS patients, and more research of its pathogenesis is required for the earlier treatment of MDS/AML patients. One of the key mechanisms of leukemic transformation of MDS overt AML is Tgfβ pathway, which has been widely and deeply studied for the elucidation of solid tumors and hematological malignancies.

We previously reported that MDS model mouse induced by C-terminal–truncating *ASXL1* mutations (*ASXL1*-MTs) is characterized with multi-lineage myelodysplasia and pancytopenia, and *SETBP1*-MT, as a second-added mutation, caused predisposition of MDS into AML and *in vitro* augmented the differential block induced by *ASXL1*-MT and inhibited apoptosis. It was also found by RNA-seq and GSEA that in the Bone Marrow (BM) cells from this AML mice, tumor growth factor-β (Tgfβ)-pathway-related genes, including *Tgfβr1*, *Tgfβr2*, *Tgfβr3*, *Smad1*, *Smad3*, and *Smad4* were globally and strongly repressed and this significant downregulation may play a part in the acquisition of leukemogenesis. The mechanism of the downregulation of Tgfβ-pathway in the leukemia induced by the combination of *ASXL1* and *SETBP1* mutations *(ASXL1*-MT/*SETBP1*-MT) has been poorly understood.

Activation of the Tgfβ pathway delays leukemogenesis

At first, to confirm the relationship of Tgfβ pathway and leukemogenesis, we performed the growth assay of BM cells of AML model mouse induced by *ASXL1*-MT/*SETBP1*-MT (cSAM: combination of *AXSL1* and *SETBP1* mutations) and found that the relative growth rate of cSAM cells transduced with the mutated *Tgfβr1* (*ALK5*(*TD*)), an active form of *Tgfβ receptor1*, is lower than cSAM cells transduced with the control vector ($p<0.001$). By contrast, the growth rate of 32D cells transduced with the mutated *Tgfβr2* (dnTGFBR2), a dominant negative form of *Tgfβr2*, is higher than with the control vector ($p=0.001$). These results indicated that the activation of Tgfβ signal pathway contributed to the repression of cSAM cell growth *in vitro*.

In order to examine whether the activation of the Tgfβ pathway is effective for repression of leukemogenesis *in*

vivo, we next transplanted cSAM cells transduced with *ALK5(TD)* into lethally radiated mice (n=4). These mice also died of acute leukemia with hepatosplenomegaly in the end, but the proportion of AML cells in bone marrow of these mice increased more slowly at the time of 16 days after the transplantation (p=0.0027), and their median survival, 26-days is significantly longer than 20-days, the median survival of AML mice transplanted with the same number of cSAM cells (p=0.0069). This indicated that the activation of the Tgfβ pathway mitigated leukemogenesis lead by *ASXL1*-MT/*SETBP1*-MT *in vivo* as well.

An HDAC inhibitor, SAHA (vorinostat) acetylates H3 and H4 which is deacetylated in cSAM cells

Generally, acetylation of histone protein is known to make the chromatin more flexible electrically and to lead a transcriptional activation. Given that our previous experiment showed that the expression of Tgfβ-pathway-related molecules, including *Tgf*βr*1, Tgf*βr*2, Tgf*βr*3, Smad1, Smad3,* and *Smad4* were repressed more significantly in AML mice induced with *ASXL1*-MT/*SETBP1*-D868N compared to MDS mice induced with *ASXL1*-MT alone, we assumed that histone acetylation levels around their promotors might be repressed in the AML mice. For confirming this possibility, we carried out chromatin immunoprecipitation (ChIP) assays with c-kit$^+$ cSAM cells. We detected the decrease of acetylation levels in H3K14, H4K5, and H4K8 at the vicinity of transcription starting sites (TSSs) of some of the Tgfβ-related genes, but not in H4K12. We next performed Western blotting and qRT-PCR, showing that an HDAC inhibitor, SAHA (vorinostat), recovered the histone acetylation state repressed in cSAM cells and expressions of Tgfβ-pathway-related molecules. These results indicated that the hypoacetylated state of histones in the AML mice is achieved by a histone deacetylase (HDAC) whose function is inhibited by SAHA.

Interestingly, SAHA was also shown to decrease the expression of *Hoxa9* and *Hoxa10*, which is known to be enhanced by SETBP1 protein functioning as a transcriptional activator, and to upregulate the self-renewal potential in myeloid neoplasms, as well as our AML model mouse with *AXSL1*-MT/*SETBP1*-MT.

An HDAC inhibitor, SAHA, ameliorates the activity of P300 protein binding to the Tgfβ**-related genes.**

P300 protein, a histone acetyltransferase, is an enzyme and regulates genes transcription via chromatin remodeling. We next carried out the ChIP assays with c-kit$^+$ bone marrow cells from healthy (Ly5.1) mouse and c-kit$^+$ cSAM cells and detected that around the TSSs of Tgfβ-related genes, P300 was less detected in cSAM cells compared to bone marrow cells from healthy mouse, and more detected in cSAM cells with SAHA compared to cSAM cells without SAHA. This suggests that P300 protein is kept away from the TSS by a HDAC complex, and can approach the TSS in the condition that SAHA avoids HDAC, or that some INHAT (inhibitor of acetyltransferases) complex binds to histones and masks them from being acetyltransferase substrates. In either case, the result is consistent with that acetylation level around the TSSs of Tgfβ-related genes are more increased in cSAM cells with SAHA compared to without SAHA.

HDAC inhibitor can be an effective treatment drug for myeloid leukemia with *ASXL1*-MT and *SETBP1*-MT.

Several papers about HDAC inhibitors as treatment drugs for leukemia have been reported before, especially Trichostatin A (TSA), 4-PBA (4-phenelbutyric acid), and SAHA. Given that deacetylation of histones around TSS of Tgfβ-related genes cause the repression of those genes and lead the leukemic transformation, we examined the efficacy of HDAC inhibitor, SAHA, for treating leukemia induced by *ASXL1*-MT/*SETBP1*-MT. As expected, SAHA attenuated the growth rate of

cSAM cells in the presence of stem cell factor and IL-3, and the colony forming capability. To confirm the efficacy of HDAC inhibitors *in vivo*, we carried out two independent experiments. First, we transplanted cSAM cells being treated in 1 μM SAHA for 7 hours into Ly5.2 mice without an irradiation. All of these mice eventually developed serious myeloid leukemia, however their overall survival were significantly longer than the mice transplanted with cSAM cells without the pre-transplantation treatment by SAHA although the numbers of transplanted cells are same. Next we orally administrated vorinostat (SAHA) into the AML mice induced by *ASXL1*-MT/*SETBP1*-MT every day for 7 days from 7 days post transplantation. Vorinostat treatment appeared to prolong the survival of the recipient mice although the difference was not statistically significant. It seems plausible to hypothesize that SAHA acetylated the histones of Tgfβ-related genes and their expressions are ameliorated, attenuating the self-renewal capability of myeloid progenitors. Collectively, these results suggest that HDAC inhibitor may play an important role as a therapeutic strategy for treating acute myeloid leukemia with *SETBP1*-MT or with a down-regulation of Tgfβ-related genes.

Hypothesis concerning the mechanism of histone deacetylation of Tgfβ-related genes and of down-regulation of *Hoxa9* and *Hoxa10* by SAHA.

We discovered that the histones around the TSSs of Tgfβ-related genes were deacetylated, depressing the genes expressions in cSAM cells, but the mechanism underlying the histone deacetylation was not elucidated completely yet. It is plausible to hypothesize that SETBP1 may recruit a HDAC to the TSSs of Tgfβ-related genes in the same way that Setbp1 reportedly recruits HDAC1 to *Runx1* promotor in myeloid leukemia with *Setbp1* overexpression, and that HDAC inhibitor, SAHA, may avoid the HDAC from the TSSs, resulting in the cancellation of histone deacetylation. From the result that P300 (HAT) are also not found around the TSSs of Tgfβ-related genes and SAHA treatment allows the HAT complex to exist, we should predict that the full-length of SET oncoprotein (INHAT) stabilized by SETBP1-MT may bind to and mask the promotor areas of Tgfβ-related genes to keep P300 (HAT) away from the genes, and that at the same time SETBP1 may exist there as an united form of SET-SETBP1-HDAC, recruiting the HDAC to the genes nearby. In this hypothesis, SAHA (HDAC inhibitor) treatment can keep the HDAC and SET-SETBP1 away at the same time from Tgfβ-related genes in a chain reaction since SET-SETBP1-HDAC1 complex may function as a unit, and P300 (HAT) can approach the genes as SET (INHAT) is away. Based on this idea, HDAC inhibitor, SAHA, can also avoid SETBP1 from binding to the genes, and this may explain the repressed expressions of *Hoxa9* and *Hoxa10* by SAHA since SETBP1 reportedly activates their expression as a transcription factor.

In conclusion, we report two mechanisms of leukemic transformation of *ASXL1*-mutated MDS driven by *SETBP1* mutation, including histone deacetylation of TSSs of Tgfβ-related genes and significant up-regulated expression of *Hoxa9* and *Hoxa10* by SETBP1 functioning as a transcriptional activator. The fact that SAHA, a HDAC inhibitor, has an opposing effect for the cell growth *in vitro* and leukemic transformation *in vivo* reinforces our hypothesis that the histones around Tgfβ-related genes are deacetylated by HDAC protein brought by SETBP1. Further research is essential for a better understanding of leukemic transformation induced by *SETBP1* mutation.