論文の内容の要旨

Leukemia-associated mutations of DNMT3A inhibit differentiation of hematopoietic stem cells and promote leukemic transformation through aberrant recruitment of Polycomb repressive complex 1.

(DNMT3A 変異はポリコーム抑制複合体 1 との異常な協調関係を通して造血幹細胞の分化阻害および白血病性形質転換の促進をもたらす。)

古屋　淳史

The mutational landscape of hematological malignancies by the recent and rapid progress in next generation sequencing has offered a refined set of risk stratification. Somatic mutations in epigenetic factors such as DNMT3A, TET2, IDH1/2, and ASXL1 exemplify the importance of mutational analysis especially in cytogenetically normal acute myeloid leukemia (CN-AML). It goes without saying that the need is heading for the development of new therapies specific for these mutated proteins, so is for the precise molecular mechanisms involving oncogenesis. DNMT3A, a member of DNA methyltransferases, is known to be mutated in about 20% of CN-AML and R882 of DNMT3A is a hot spot of mutation. Despite cumulating clinical data have emphasized the importance of DNMT3A mutation as a poor prognostic factor, little is known about how DNMT3A mutation contributes to leukemogenesis in vivo. DNA methyltransferases catalyze DNA methylation on the cytosine of CpG dinucleotides and, in particular, DNMT3A and DNMT3B are the main players involved in de novo methylation, and their deficiency deprives embryonic stem cells of differentiation potential. R882, the most prevalent mutated site is located in the catalytic domain of DNMT3A. It has recently reported that DNMT3A R882 mutant causes loss of DNA methyltransferase activity in vitro. On the contrary to the recent report showing that DNMT3A-mutated AML samples have an apparent DNA hypo-methylation signature, there are no distinct gene expression profiles regarding DNMT3A mutation. As an epigenetic factor, DNMT3A is well known to interact with several histone modifiers including polycomb group proteins (PcG) to suppress their target genes' transcription. The functional cooperation between DNA methyltranferase and PcG is considered to be responsible for cancer development. In this study, I aimed to elucidate the impact of DNMT3A mutation on the development and maintenance of AML, including molecular mechanisms other than DNA methylation.

Firstly, I investigated whether DNMT3A wild-type (WT) and R882 mutant could affect proliferation of human AML cell lines. I lentivirally transduced empty vector (EV), DNMT3A WT, or R882H to HL-60 cell line which do not carry DNMT3A mutation. DNMT3A WT-transduction resulted in a decreased proliferation of HL-60 cells relative to EV and R882H-transduction. While DNMT3A WT promoted

differentiation of HL-60 cells, R882H-transduced HL-60 cells remained immature. In addition, DNMT3A R882H-transduced HL-60 cells are resistant to ATRA-induced neutrophil differentiation compared to EV-transduced HL-60 cells. These results indicated that DNMT3A R882 mutant induced differentiation block and ATRA resistance to HL-60 cells, which is a striking difference from DNMT3A WT.

Next, I investigated the effects of exogenous expression of DNMT3A R882 mutant in hematopoiesis by transducing 5-FU primed mouse bone marrow (BM) cells with retroviral EV-, DNMT3A WT-, R882H-, or R882C-IRES-EGFP. I sorted GFP+ cells, then transplanted to lethally irradiated mice for assessing the hematopoietic repopulating capacity of these cells in vivo. I sacrificed these transplanted mice at four weeks post-transplant and investigated whether exogenous expression of DNMT3A WT and R882 mutant affected BM stem/progenitor frequency. Interestingly, while the frequency of long-term HSC (LT-HSC; lineage- (L), Sca1+ (S), c-kit+ (K), CD150+, CD48-) was increased in recipients of R882 mutant-transduced cells compared to recipients of EV- and DNMT3A WT-transduced cells. These results indicated that exogenous expression of DNMT3A R882 mutation contributed to aberrant HSC accumulation.

In order to identify the down-stream target genes of mutant DNMT3A that evoked HSC accumulation, I sorted GFP+LSK cells from transplanted mice at four weeks post-transplant and conducted quantitative PCR experiment to assess the expression of various hematopoiesis-related genes. DNMT3A R882 mutant-transduced LSK cells showed higher Hoxb2 and Hoxb4 expression which are known to be highly expressed in clinical AML samples harboring DNMT3A mutation and associated with murine HSC self-renewal. Conversely, myeloid differentiation-associated genes, including PU.1 and Cebpα, were down-regulated in DNMT3A mutant-transduced LSK cells. Targeted bisulfite sequencing of these cells showed hypomethylation of the Hoxb2 promoter-associated CpG island in DNMT3A R882 mutant-transduced cells, which was consistent with the alteration in Hoxb2 mRNA expression of transplanted mice. On the other hand, DNMT3A R882 mutant caused no change in methylation status of PU.1 promoter-associated CpG island, indicating that DNA methylation-independent mechanism underlies PU.1 downregulation.

Given that DNMT3A interacts with several histone modifiers to regulate target gene transcription, we performed co-immunoprecipitation to investigate whether these interactions are altered by DNMT3A mutation. Interestingly, DNMT3A R882H and R882C mutant proteins exhibited augmented interaction with BMI1 and MEL18 which are key components of polycomb repressive complex 1 (PRC1). In addition, RING1B, an essential component of PRC1, was more firmly bound to DNMT3A mutants than WT, irrespective of the type of amino acid substitution. Previous reports have revealed that PRC1 complexes are divisible into two distinct types. One is PRC2-dependent PRC1 complex (PDPC) and the other is PRC2-independent PRC1 complex (PIPC). PDPC represses the PRC2-targeted gene expression through chromatin compaction and placement of a repressive mark, histone H2AK119 ubiquitination. Western blotting analysis revealed that DNMT3A R882 mutant (especially in R882H) had greater affinity

with CBX7 (representative for PDPC) than WT, but there showed no altered interaction between RYBP (representative for PIPC) and DNMT3A R882 mutant compared to WT. These observations suggested that PDPC favored DNMT3A R882 mutant over DNMT3A WT as a collaborating partner.

To assess the importance of the PRC1 complex interaction in DNMT3A R882 mutant-induced aberrant HSC accumulation, I performed transplantation assay using Bmi1 heterozygous knockout mice. I transplanted EV, WT, or R882H-transduced c-kit+ $Bmi1^{+/+}$ or $Bmi1^{+/-}$ mouse BM cells to lethally irradiated mice and analyzed these mice at four weeks post-transplant. Strikingly, Bmi1 heterozygosity canceled the effect of DNMT3A R882 mutation in terms of the frequency of LT-HSC without influence on EV-transduced HSC. Furthermore, heterozygosity of Bmi1 restored the expression levels of down-regulated myeloid differentiation-associated genes (especially PU.1) to normal levels in R882 mutant-transduced LSK cells, while Hoxb cluster genes were up-regulated, irrespective of Bmi1 genotype. These results suggested that augmented interaction between DNMT3A R882 mutant and PRC1 complex plays an important role in R882 mutant-induced HSC accumulation through transcriptional silencing of differentiation-associated genes.

To assess whether PU.1 is a critical effector on DNMT3A mutant, I performed second transplantation assay using murine PU.1 expression vector. EV or R882H-transduced c-kit+ cells sorted from primary transplanted mice were transduced with EV or PU.1 and these cells were transplanted to lethally irradiated mice, which resulted in that R882H-transduced stem/progenitor cells had a higher repopulating capacity and exogenous PU.1 expression impaired a repopulating capacity in both EV and R882H-transduced cells to the similar level. To analyze whether PU.1 is a direct target of increased interaction between DNMT3A R882 mutant and PRC1 complex, I performed chromatin immunoprecipitation (ChIP)-qPCR experiment using HL-60 cells transduced with DNMT3A WT or R882H mutant. ChIP-qPCR showed that BMI1 and RING1B were more efficiently recruited to the upstream regulatory element of PU.1 upon expression of DNMT3A R882H than WT, while the amount of DNMT3A recruited were comparable between DNMT3A WT and R882H. In addition, RING1B-mediated lysine 119 ubiquitination on histone H2A, a repressive mark, was more enriched at the same locus in R882H mutant-transduced HL-60 cells.

Then I searched for some potent collaborating genes with DNMT3A R882 mutant required for development of AML. Focusing on HOXA9, I identified that DNMT3A R882 mutation promotes blastic transformation of murine c-kit+ BM cells in combination with HOXA9 in vitro. Morphological and surface marker analysis revealed that these cells were F4/80+ monocytic blasts, consistent with clinical observation that DNMT3A mutation is frequently found in FAB M4/M5 myelomonocytic/monocytic AML. These results indicated a potential collaboration between DNMT3A R882 mutant and HOXA9 in malignant monoblastic transformation of hematopoietic cells. Subsequently, I assessed the impact of PRC1 complex on this monoblastic transformation using Bmi1 heterozygous knockout mice. Interestingly, Bmi1 heterozygosity impaired this monoblastic transformation of R882H and HOXA9 co-transduced

progenitors.

In order to investigate the importance of augmented interaction between PRC1 complex and DNMT3A R882 mutant for maintenance and ATRA-resistance of DNMT3A-mutated AML, I retrovirally transduced shRNAs targeting RING1B to HL-60 cells transduced with DNMT3A WT or R882H. As expected, knockdown of RING1B eliminated the distinction between WT- and R882H-transduced HL-60 cells in proliferation capacity and between R882H-non expressed and R882H-expressed cells in sensitivity to ATRA. Taken together, these results demonstrated that PRC1 complex is required for monoblastic transformation of normal murine BM progenitors, for maintenance of differentiation block capacity, and for resistance to differentiation-inducing therapy in DNMT3A-mutated AML cells.

Based on my findings, I propose that DNMT3A R882 mutant has two distinct mechanisms for leukemogenesis: (1) DNA methylation-dependent effect, leading to DNA hypo-methylation; (2) DNA methylation-independent effect, recruiting the PRC1 complex to specific target loci. Especially, as an indicator of DNA methylation-independent mechanism, my data indicate that DNMT3A R882 mutation-mediated recruitment of PRC1 complex is associated with transcriptional silencing of differentiation-associated genes, blocking differentiation of HSCs and leukemic cells. Attenuating the functional cooperation between DNMT3A mutant and PRC1 complex could be a promising therapeutic target in DNMT3A-mutated hematological malignancies.