

# Functional analysis of ASXL1 mutation in myeloid malignancies

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博士論文(要約)

Functional analysis of ASXL1 mutation in myeloid malignancies  
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(骨髄系腫瘍における ASXL1 変異の機能解析)

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Recent progress in whole genome sequencing has identified recurrent somatic mutations in a gene encoding additional sex combs like 1 (*ASXL1*), in a variety of myeloid malignancies. The impact of *ASXL1* mutations has been further emphasized by their association with poor prognosis regardless of disease type. In addition, mutations of *ASXL1* have been detected in blood cells of individuals without apparent hematological malignancies, a supposedly pre-malignant condition called age-related clonal hematopoiesis or clonal hematopoiesis of indeterminate potential. These kinds of clonal hematopoiesis are associated with expansion of altered clones and increased risk of developing hematological neoplasia, further underscoring the importance of *ASXL1* in constituting pre-malignant conditions. However, the precise molecular mechanisms of mutated *ASXL1* are largely unknown, mainly due to lack of appropriate in vivo model for analysis. Here, we generated a constitutive knock-in (KI) mouse model with endogenous expression of mutant *Asx1l*, and analyzed the biological effect of *Asx1l* mutation with this model.

To extensively investigate the biological effects of somatic expression of the *Asx1l* mutant, we first generated a murine model for endogenous expression of the *Asx1l* G643WfsX12 mutation from the endogenous locus of *Asx1l*. Genotypic analysis of live births revealed that not only *Asx1l*<sup>G643fs/G643fs</sup> but also *Asx1l*<sup>G643fs/+</sup> newborns were underrepresented, suggesting partial embryonic lethality of heterozygous and homozygous embryos. *Asx1l*<sup>G643fs/+</sup> mice showed hypomorphic features including lower body weight, though no apparent facial features or malformations were observed. These data indicate that germline *Asx1l* mutant leads to a dysmorphic phenotype resembling clinical manifestations of Bohring-Opitz syndrome with de novo constitutive *ASXL1* mutation.

We next examined whether heterozygous *Asx1l* mutant knock-in causes manifestations of myeloid malignancies in vivo. We used both young mice (3 months old) and old mice (12 months old) from each genotype (*Asx1l*<sup>G643fs/+</sup> and WT), because incidence of MDS considerably increases with age. There were no significant differences in bone marrow (BM) cellularity and spleen weight between WT and *Asx1l*<sup>G643fs/+</sup> mice of young or old age group. However, Wright-Giemsa-stained peripheral blood cells

from old *Asx11*<sup>G643fs/+</sup> mice showed dysplastic features, including bilobed and hypo-segmented neutrophils with fine nuclear bridging consistent with pseudo-Pelger-Huët anomaly, hyper-segmented neutrophils, and apoptotic neutrophils. These features were not found in young *Asx11*<sup>G643fs/+</sup> mice. Peripheral blood (PB) counts of these young *Asx11*<sup>G643fs/+</sup> mice did not show any abnormalities except for thrombocytosis. However, leukopenia and thrombocytosis were observed in old *Asx11*<sup>G643fs/+</sup> mice. Flow cytometric analyses of *Asx11*<sup>G643fs/+</sup> BM cells confirmed increased frequency of granulocytic/monocytic cells together with decreased frequency of B cells. In order to determine whether physiological expression of *Asx11* mutant affects hematopoietic stem and progenitor cell (HSPC) pool in vivo, we next analyzed the frequency of Myeloid progenitor (MP), c-kit<sup>+</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> (LSK) and the more purified HSC fraction of c-kit<sup>+</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> CD34<sup>-</sup> CD135<sup>-</sup> (LT-HSC) cells. The proportions of all these fractions were decreased in *Asx11*<sup>G643fs/+</sup> mice compared with WT controls. When the each myeloid progenitor subset was analyzed within the MP populations, the percentage of granulocyte-monocyte progenitors (GMP) cells was significantly increased. The proportions of common myeloid progenitors (CMP) and megakaryocyte-erythroid progenitor (MEP) cells were comparable between the genotype. These data suggest that *Asx11* mutant decreases the HSPC pool and alters distribution of myeloid progenitor cells in young mice.

To investigate the mechanism of decreased HSC pool in *Asx11*<sup>G643fs/+</sup> mice, we examined apoptotic status of BM cells from *Asx11* mutated mice. The apoptosis assay revealed significantly increased apoptosis in LSK and GMP fractions of freshly isolated *Asx11*<sup>G643fs/+</sup> BM cells compared with WT littermate controls. Collectively, these data show that *Asx11* mutant leads to a mild phenotype in mice resembling clinical manifestations of human myelodysplastic syndrome (MDS).

Interestingly, after long follow-up for up to two years, *Asx11*<sup>G643fs/+</sup> mice became moribund while age-matched littermate control mice were still alive. Those mice developed anemia, leukocytosis and neutrophilia/monocytosis. BM cellularity of the *Asx11*<sup>G643fs/+</sup> mice was significantly increased compared with WT mice, and splenomegaly was observed only in the *Asx11*<sup>G643fs/+</sup> mice. The BM cells isolated from these old *Asx11*<sup>G643fs/+</sup> mice showed dysplastic neutrophils and monocytes. Histologic analysis of spleen and liver showed disrupted splenic architecture, with an increased frequency of myeloid cells, and perivascular infiltration of myeloid cells in the liver. The presence of abnormality of peripheral blood count, myeloid dysplasia and histological findings were consistent with human MDS/Myeloproliferative neoplasm (MPN), especially chronic myelomonocytic leukemia (CMML). However, no mice exhibited any signs of acute leukemia after two-year observation, suggesting that physiological expression of *Asx11*<sup>G643fs</sup> mutant alone is insufficient to induce leukemic transformation in vivo, characterized by complete differentiation block.

In order to evaluate the self-renewing and repopulating capacity of HSPCs, we performed in vitro colony-forming cell assay for short-term analysis, and in vivo competitive repopulation assay for long-term analysis. *Asx11*<sup>G643fs/+</sup> cells had increased colony-forming capacity, and these cells could be replated up to fifth round, which was longer than WT control cells, though no sign of immortalization was observed. In contrast, in the competitive repopulation assay with BMT model, no significant difference was observed in the donor chimerism of each lineage between *Asx11*<sup>G643fs/+</sup> and WT cohort. Considering that *Asx11*<sup>G643fs/+</sup> mice show decreased phenotypically-defined HSPCs, the enhanced replating potential during serial passages in methylcellulose culture and comparable repopulating activity in vivo might suggest that *Asx11*<sup>G643fs/+</sup> HSPCs are functionally altered, i.e. increased progenitor activity with partial differentiation block of myeloid lineage cells.

To understand the basis of the decreased HSC pool and aberrant myeloid differentiation in *Asx11*<sup>G643fs/+</sup> mice, we performed RNA-sequencing (RNA-seq) to comprehensively analyze the expression profiles of sorted LSK cells from young *Asx11*<sup>G643fs/+</sup> mice and age-matched controls. Analysis of RNA-seq data identified differentially expressed genes in *Asx11*<sup>G643fs/+</sup> LSK cells (52 genes), including 25 upregulated genes and 27 downregulated genes. The upregulated genes included genes associated with regulation of apoptosis and cell aging, consistent with our prior in vivo experiments.

We next performed gene set enrichment analysis (GSEA) to identify gene sets enriched in LSK cells from *Asx11* KI mice. We identified derepression of BMI1 and EZH2 target genes. BMI1, together with RING1, is a member of core complex of PRC1, and EZH2 is a well-known component protein of PRC2. We first investigated the expression of posterior *Hoxa* cluster genes and H3K27me3 level at the promoter regions of these genes. These genes were significantly upregulated in the BM cells from *Asx11*<sup>G643fs/+</sup> mice. However, decreased levels of H3K27me3 at the promoter regions were not observed.

It is well known that senescence-related genes are regulated by BMI1, a primary component of PRC1 complex. Young *Asx11*<sup>G643fs/+</sup> mice share some features with normally aged mice in that they show myeloid-skewed cell differentiation. Based on these common phenotypes and the result of GSEA, we next examined the expression of senescence-related genes regulated by BMI1. Interestingly, senescence-related *p16Ink4a*, *p21*, and *p15Ink4b* genes were all upregulated in the BM cells from *Asx11*<sup>G643fs/+</sup> mice. Along the same line, repressive histone mark H2AK119ub1 at the promoter region of *p16Ink4a* was decreased.

It is well known that most of *ASXL1*-mutated patients with myeloid malignancies have other concurrent mutations at diagnosis. Among several recurrent mutations co-occurring with *ASXL1* mutations, we focused on *IDH1* mutation. *IDH1* gene encodes isocitrate dehydrogenases and frequently mutated in glioblastomas and cytogenetically normal acute myeloid leukemia (AML). *IDH1* mutations typically

produce mutant enzymes with aberrant activity, thus human AML cells with this mutation show global DNA hypermethylation. From our data, *Asx1l* mutant alone is insufficient to induce AML, thus we examined whether truncated form of *Asx1l* would collaborate with *IDH1* mutant in murine BMT model. Similar to *ASXL1*, *IDH1*<sup>R132H</sup> alone does not induce any hematological abnormalities as reported, and the presumptive role of *IDH1*<sup>R132H</sup> is a cooperating factor in disease progression, not in disease initiation. In this model, transduction in *Asx1l*<sup>G643fs/+</sup> cells, but not in *Asx1l* WT cells, with *IDH1*<sup>R132H</sup> induced lethality in mice after a long latency. Recipient mice reconstituted with *Asx1l*<sup>G643fs/+</sup> cells developed leukocytosis, anemia and hematosplenomegaly. The Wright-Giemsa-stained BM cells isolated from *Asx1l*<sup>G643fs/+</sup>-derived mice were mostly blasts, indicating AML progression. Flow cytometric analyses of *Asx1l*<sup>G643fs/+</sup>-derived BM cells confirmed proliferation of leukemic cells expressing GMP-like cell surface markers. All of serial transplants with *Asx1l*<sup>G643fs/+</sup>-derived AML cells developed AML with shorter duration, showing their high leukemogenic potential. These data indicate that mutant form of *Asx1l* induce AML in cooperation with *IDH1*<sup>R132H</sup>.

Collectively, the present study demonstrates that endogenous expression of *Asx1l* mutant in mice is sufficient to recapitulate human MDS and MDS/MPN, and promote leukemogenesis with a cooperating factor, implying oncogenic activity of *Asx1l* mutant. Given the paucity of mouse models of human MDS and MDS/MPN based on endogenous expression of recurrent somatic mutations, I believe that this model provides an ideal platform for not only unveiling the molecular basis for MDS and transformation to AML, but also developing novel therapies for MDS patients.