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Gene mutations of acute myeloid leukemia in the genome era

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Abstract Ten years ago, gene mutations found in acute myeloid leukemia (AML) were conceptually grouped into class I mutation, which causes constitutive activation of intracellular signals that contribute to the growth and survival, and class II mutation, which blocks differentiation and/or enhance self-renewal by altered transcription factors. A cooperative model between two classes of mutations has been suggested by murine experiments and partly supported by epidemiological findings. In the last 5 years, comprehensive genomic analysis proceeded to find new gene mutations, which are found in the epigenome-associated enzymes and the molecules never noticed so far. These new mutations apparently increase the complexity and heterogeneity of AML. Although a long list of gene mutations might have been compiled, the entire picture of molecular pathogenesis in AML remains to be elucidated because gene rearrangement, gene copy number, DNA methylation and expression profiles are not fully studied in conjunction with gene mutations. Comprehensive genome research will deepen the understanding of AML to promote the development of new classification and treatment. This review focuses on gene mutations that were recently discovered by genome sequencing.

Keywords Acute myeloid leukemia · Chromosome translocation · Gene mutation · Genome sequencing · Epigenetics · Prognosis

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Introduction

Acute myeloid leukemia (AML) is a neoplasm of the clonal and irreversible expansion of myeloid blasts and most common among adult acute leukemia. It is heterogeneous regarding clinical feature, morphological and immunophenotypic features, and karyotypic and genetic abnormalities [1, 2]. Initial molecular biological studies started with cloning recurrent chromosomal translocations; *RUNX1-RNNX1T1*, *CBFB-MYH11*, *PML-RARA*, and *MLL* gene rearrangement from t(8;21), inv(16), t(15;17) and 11q23 translocations, respectively. These studies presented a paradigm of leukemogenesis. The translocations generate chimeric transcriptional factors, which alter expressions of genes critical for hematopoietic development and/or differentiation. In vitro and in vivo experiments support that these abnormalities block hematopoietic differentiation and/or promote self-renewal, while their transgenic mice developed AML with low frequency except *MLL-AF4* and *MLL-AF9* [3–7]. In addition, critical transcriptional factors for myeloid differentiation, *CEBPA* and *RUNX1*, are point-mutated in AML without above translocations [8–10]. Importantly, this class of mutations is closely associated with morphology, phenotypes and prognosis of AML and is mutually exclusive [11].

On the other hand, classical transformation assay revealed *NRAS* or *KRAS* mutation in AML as well as solid tumors [12, 13]. Thereafter, the receptor tyrosine kinase-encoding genes, *FLT3* and *KIT*, were found to be frequently mutated in AML [14–20]. A receptor tyrosine kinase (RTK)-RAS-MAPK pathway is one of the most important intracellular signaling in myeloid malignancies including myeloproliferative neoplasm (MPN) and myelodysplastic syndrome (MDS). In vitro and in vivo studies suggest that this class of mutations augments proliferation and survival

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signals, but does not induce AML but MPN by itself in murine model [21]. Clinically, this type of mutations sometimes emerge or occasionally disappear during clinical course of AML and MDS [22–24]. These mutations (so-called class I mutation) are mutually exclusive but not so strictly as the above class of mutations (class II mutation), and not related with AML subtypes except for the preference of *KIT* mutation to CBF leukemia.

Simultaneous expression of two classes of mutations, i.e. *PML-RARA* and *FLT3*-ITD, in murine hematopoietic progenitor cells caused AML with high frequency and the following similar experiments suggested that two classes of mutations work synergistically to develop AML in vivo [6]. The cooperating model does not only fit with the murine experiments, but also with epidemiological findings in human AML, providing the backbone for the WHO classification in AML [25]. This model is also used to characterize the new gene mutation. *NPM1* mutation which was found in 2005 and the functional significance not elucidated at that time are exclusive to class II mutations and frequently overlaps with class I mutations, suggesting that it belongs to class II mutation [26, 27]. However, it is often difficult to dissect functions of class I and class II mutations [7]. For example, a representative gene mutation of class I, *FLT3*-ITD, does not only mediate proliferation and anti-apoptosis signals, but also block differentiation through down-regulating C/EBPα and PU.1 [28–30].

Year 2009 is the first year of leukemia genome. *TET2* located on 4q24 was identified as a target of deletions or mutations in myeloid malignancies [31, 32]. *IDH1/2* mutation was found by whole-genome sequencing of a primary, cytogenetically normal AML (CN-AML) [33]. In the next year, *DNMT3A* and *EZH2* were recognized to be mutated by genome-based methods [34, 35]. Either of these new mutations directly targets epigenetic control such as DNA/histone methylation status [36]. In addition to them, mutations were found in transcriptional corepressors, ASXL1, BCOR and BCORL1 [37–39]. In 2012, cohesion complex-encoding gene mutation was reported [40]. Most these mutations were generally identified in all types of myeloid malignancies, while discovery of frequent mutations of splicing machinery, which is highly associated with myelodysplasia and the formation of ring sideroblasts, is important to clarify disease type- and/or phenotype-specific pathological roles of gene mutations [41, 42].

Although more years are needed to draw the whole picture of AML gene abnormalities, we may have obtained a list of recurrently mutated genes in AML (Table 1). Although all the mutations are likely to be unrelated at first glance, if looking at its features, they are found to be related each other. In Fig. 1, we propose a pathway map, in which mutated gene products in AML are functionally connected. In this figure, we make a point not to draw uncontrolled cell-cycle and cell survival which are secondary operations from mutated gene products. In the case of acute lymphoblastic leukemia and lymphoma, genes associated with cell-cycle and cell survival are frequently targeted, suggesting that the mutational profiles depend on its cellular context. Accordingly, it may be possible to highlight that AML is a disease of transcription, in another word, differentiation.

Next, we summarize gene mutations found in AML during these 5 years.

Mutations in epigenetic modifiers

TET2

The TET family proteins (Tet1, Tet2, Tet3) are catalyzing enzymes 5-methylcytosine (5-mC) to hydroxymethylcytosine (5-hmC), which have been shown as an initial process of DNA demethylation [43–45]. Mutations of the *TET2* gene have been found in various myeloid malignancies; 8–27 % of AML, 20–25 % of MDS, 4–13 % of MPN [31, 32, 46–49]. *TET2* mutations are loss-of-function of mono allele in most cases, including missense, frameshift and nonsense mutations. Importantly, *TET2* mutations are almost mutually exclusive with *IDH1/2* mutations, suggesting a similar epigenetic defect as *IDH1/2* mutations [36, 50].

The base 5-hmC is recently suspected as a new epigenetic marker in addition to being an intermediate metabolite during cytosine demethylation. It has been suggested that 5-hmC is associated with transcriptional regulation for promoter and enhancer functions [51–53]. In vivo, TET2 inactivation induced in hematopoietic abnormalities predisposing not only myeloid but also lymphoid malignancies [54]. However, the precise mechanisms and downstream effects of TET2 are yet unknown.

The prognostic relevance of *TET2* mutations remains controversial. Some studies suggest an adverse impact of *TET2* mutations on outcome in certain AML subgroups; in other studies, no prognostic significance was found [47–50].

DNMT3A

DNA methyltransferase (DNMT) family enzymes transfer a methyl radical to DNA, and in human, DNMT3A and 3B are methyltransferases that convert cytosine to 5-methylcytosine and generate de novo DNA methylation, whereas DNMT1 mediates maintenance methylation [55].

Mutations of *DNMT3A* are reported in 18–23 % of AML, including 20–35 % with normal karyotype, 8 % of MDS and 7–15 % of MPN [34, 36, 50, 56–58]. The locus

Table 1 Recurrently mutated genes in AML

Function	Gene	Mutation frequency	Grouping
Tyrosine kinase	FLT3	ITD: 20–28 %	Class I
		KDM: 5–10 %	
	KIT	25–30 % in CBF-AML	
	JAK1	1–3 %	
	JAK3	1–2 %	
RAS pathway	NRAS	9–14 %	
	KRAS	5–17 %	
Protein phosphatase	PTPN11	4–5 %	
Ubiquitin pathway	CBL	2–3 %	
Nuclear-cytoplasmic shuttling phosphoprotein	NPM1	25–35 %	Class II
Transcription factor	CEBPA	10–20 %	
	RUNX1	5–13 %	
	GATA2	3–5 %	
	RUNX1-RUNX1T1	10–15 %	
	CBFB-MYH11	3–8 %	
	PML-RARA	5–10 %	
	MLL fusion	5–9 %	
	DEK-NUP214	1 %	
DNA hydroxymethylation	TET2	8–27 %	Class III? (mutations in epigenetic modifires)
	IDH1	6–9 %	
	IDH2	9–12 %	
DNA methylation	DNMT3A	18–23 %	
Histone 3 K27 methylation	EZH2	Rare in AML 8–12 % of MPN-BC	
Histone 3 K4 methylation	MLL	5–6 %	
		PTD: 5–13 %	
Histone 3 K27 tri-methylation	ASXL1	3–11 %	
Transcriptional corepressor	BCOR	4–5 %	?
	BCORL1	6 %	
Cohesin complex	STAG2	2 %	
	SMC3	3 %	
	SMC1A	3 %	
	RAD21	2 %	
Tumor suppressor	TP53	7–12 %	
	WT1	10–13 %	

of the mutations is clustered at R882 in a half of cases and distributed widely in other cases. The mutations are supposed to be loss-of-function, although it is not well characterized. Aberrant methylation patterns induced by the mutation might be associated with initiation and progression of leukemia, although the biological consequences of the mutations remain unclear [55–57, 59]. In vivo study revealed that Dnmt3a-null hematopoietic stem cells had an increased self-renewal capacity and lost their differentiation potential, which was accompanied by aberrant methylation pattern implicated in leukemogenesis [60, 61]. However, knockout of *Dnmt3a* alone was not sufficient to initiate leukemia.

DNMT3A mutations might be associated with poorer prognosis, and is frequently associated with *NPM1* and *FLT3* mutations [34, 50, 56–58]. Several different loss-of-function mutations have been found in all exons of *DNMT3A*, while a missense point mutation at amino acid R882 which decreases catalytic activity and DNA binding affinity is most frequently identified.

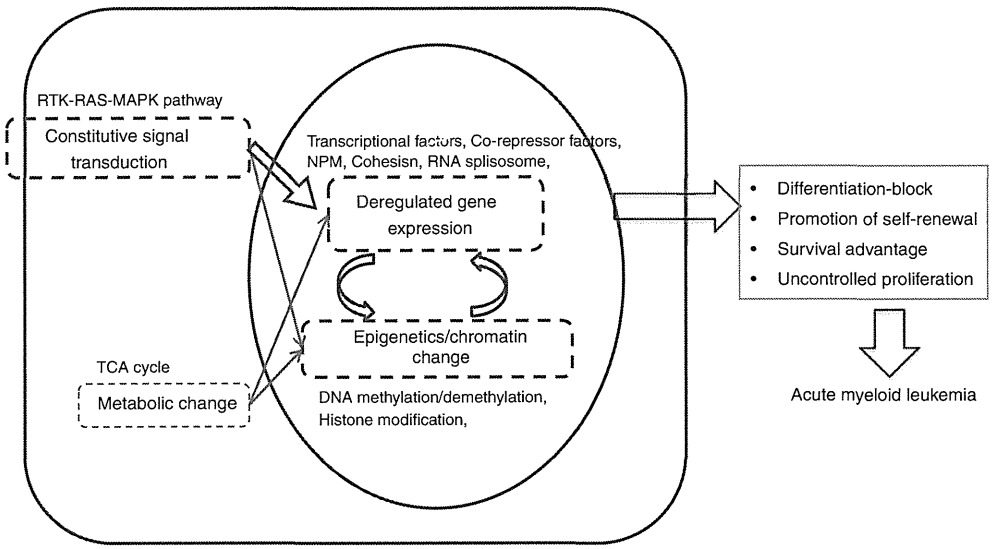

Fig. 1 A schematic diagram of intracellular signaling associated with AML. Gene mutations in AML are functionally classified into three general classes: constitutive signal transduction (class I), deregulated gene expression (class II), and epigenetics/chromatin change (class III). Three classes of mutations are functionally linked in this model, whereas according to the Gilliland's model two classes of mutation work in a cooperative or complementary manner [26]. The molecular alterations are eventually summarized toward the deregulated gene expression in molecular pathogenesis

EZH2

EZH2 is a H3K27 methylatransferase which is one of the components of polycomb repressive complex 2 (PRC2) [62]. PRC2 has been supposed to be required for silencing target genes and maintaining "stemness" in stem cells [63]. Several studies established that EZH2 is overexpressed in solid tumors, and high expression is associated with tumor progression, indicating the role of EZH2 as an oncogene in these tumors [64]. Mutation at the tyrosine 641 residue within the catalytic domain of EZH2 is recurrently identified in germinal center-type diffuse large B-cell lymphoma and follicular lymphoma [65]. Biochemical analysis revealed that Y641 mutation increases di- and tri-methylation of H3K27 in spite of impairing H3K27 mono-methylation. Furthermore, in vivo study suggested that EZH2 augments leukemogenesis by reinforcing differentiation block in AML [66]. These results are consistent with the assumption that the increasing H3K27 methylation activity of PRC2 represses the expression of tumor suppressor genes. In contrast to these data, a wide variety of loss-of function mutation of the *EZH2* gene has been found in myeloid malignancies. It is found in 6 % of MDS and 3–13 % of MPN, but extremely rare in AML [35, 36, 67]. Among them, mutations are prevalent in CMML and associated with adverse outcome [68]. The function of EZH2 remains unclear in hematopoiesis, because *Ezh2* conditional knock-out (KO) mice had minimal myeloid differentiation defects [69]. However, loss-of-function rather than overexpression of EZH2 might be involved in pathogenesis of myeloid malignancies through the insufficient H3K27 methylation status.

IDH1/2

IDH1 and IDH2 are NADP-dependent isocitrate dehydrogenases that catalyze isocitrate to α-KG in TCA cycle, and the subcellular localizations are in cytosol and mitochondria, respectively [70]. *IDH1/2* mutations are detected in 15–33 % of AML mostly in normal karyotype-AML, 3.5 % of MDS, 2–5 % of MPN, and also in glioma and multiple endochondromatosis [70–74]. The mutations have been shown to exhibit a gain-of-function leading to aberrant accumulation of 2-hydroxyglutarate (2-HG). 2-HG is an oncometabolite which inhibits an enzymatic activity of TET2 and stimulates HIF1-α, leading to initiation and promotion of cancer [75–77]. Therefore, the *IDH1/2* mutations functionally overlap with TET2 mutation, resulting in hypermethylation of leukemia cells, disrupt TET2 function, and impair hematopoietic differentiation. Recently, IDH mutation was reported to expand murine hematopoietic progenitor pool as well as altering epigenetics [76].

The impact of *IDH* mutations on survival of AML patients is unclear like the mutation of *TET2*. Some studies have observed no difference in outcome with respect to the *IDH* mutation status, while others have demonstrated a poor prognostic impact in certain AML subgroups. Recent studies suggest that the impact of *IDH2* mutation on prognosis depends on the mutation site, with the IDH2 R140 mutation being an independent favorable prognostic factor in AML patients [71].

Cohesin complex genes

Cohesin complex regulates the cohesion and separation of sister chromatids during cell division, and recently has been known to regulate gene transcription associated with cell development and differentiation [78, 79]. Cohesion complex is composed by Smc1, Smc3, Rad21 and SA1/2. SA2-encoding gene, STAG2, and RAD21 are reported to be deleted in AML genome, and recently *SMC1A, SMC3, STAG2* and *RAD21* are found to be mutated in a loss-of-function manner [40, 80, 81]. The mutations are found in a variety of AML except acute promyelocytic leukemia and are unrelated to chromosomal instability. The clinical and biological significance of cohesion complex-coding genes is to be further elucidated.

Transcriptional corepressor

BCOR

BCOR protein was identified as an interacting corepressor of BCL6, a POZ/zinc finger transcription repressor that is required for germinal center formation and may influence apoptosis [82]. Mutations in the BCOR gene have also been found to be associated with AML [38].

ASXL1

ASXL1 is involved in the maintenance of both activation and silencing of the *HOX* genes, which are involved in development of body axial structure as well as in chromatin remodeling [83]. ASXL1 also acts as a corepressor of nuclear receptors such as PPARγ and RARγ [84]. Although the KO mice of *ASXL1* have no defect in hematopoiesis [83], it has been recently reported that *ASXL1* mutants promote myeloid transformation through loss of PRC2-mediated H3K27 tri-methylation [85]. *ASXL1* mutations have been found in 3–11 % of AML, 14 % of MDS and 2–23 % of MPN. The mutation frequency is high, from 27 to 49 % in MDS/MPN including CMML [50, 86–88]. The mutations consist of missense, nonsense and frame shift, suggesting a loss-of-function. It has been also reported that *ASXL1* mutation status can change during disease evolution.

Interaction of genetic alterations in AML

Several somatic mutations are accumulated in cancer cells during the development and progression. These mutations are classified into two types of mutations: "driver" mutations that provide a selective advantage and "passenger" mutations that are simply happened without any role. In AML, it has been demonstrated both by clinical analysis and experimental models that the accumulation of two types of driver mutations (class I and class II mutations) is required for the clonal expansion of leukemia cells [7]. Therefore, it is important to evaluate whether each mutation is driver or passenger mutation and which combinations of overlap mutations are associated with the leukemogenesis and clinical significance in AML. To date, several combinations of class I and class II mutations have been apparent. Although *FLT3*-ITD and *PML-RARA*, *KIT* mutation and *RUNX1-RUNX1T1* or *CBFB-MYH11*, and *FLT3*-ITD and *NPM1* mutation are frequently identified, the other combinations are relatively infrequent. Recently, it has been suggested that a part of mutations, such as epigenetic modifiers, generates a new class (class III), because of their overlap mutations both with class I and class II mutations (Fig. 2) [36, 50].

Consistent with the biological evidence that *TET2* and *IDH1/2* mutations are functionally overlapped, these mutations are mutually exclusive in AML cells [36]. Likewise, *DNMT3A, ASXL1* and *MLL* mutations, which directly regulate methylation state of DNA and/or the histone, are exclusively identified in AML cells. Importantly, overlap mutation of *TET2* or *IDH1/2* mutation with *DNMT3A, ASXL1* or *MLL* mutation is identified in AML cells. This overlap is infrequently identified, but indicates the possible involvement of more than one molecules, which show the opposite methylation status by these epigenetic mutations, in leukemogenesis. In addition, these epigenetic mutations might contribute to pathogenesis of AML in concert with class I and/or class II mutations because of their frequent overlap mutations. Particularly, *TET2, IDH1/2* and *DNMT3A* mutations are frequently overlapped with *FLT3* and *NPM1* mutations. In contrast, *ASXL1* and *MLL* mutations, which mainly affect the histone methylation status, are exclusively mutated with class I mutations, except for the frequent overlap of *MLL*-partial tandem duplication (*MLL*-PTD) and *FLT3* mutations. Furthermore, *ASXL1* and *MLL* mutations are overlapped both with class II, such as *RUNX1* and *CEBPA*, and *TET2* or *IDH1/2* mutations [37, 87]. On the other hand, *ASXL1* mutation recurrently overlaps with *JAK2*, *RAS* and *NF1* mutations as well as *U2AF35* and *RUNX1* mutations in MDS and MPN [89, 90]. In addition, it has been demonstrated that knock down of ASXL1 collaborates with *NRAS* G12D mutation to promote myeloid transformation in vivo [85]. Since ASXL1 mutation is preferentially identified in secondary AML (30.3 %) rather than de novo AML (6.5 %), further analysis is required to clarify whether the oncogenic role of ASXL1 in de novo AML is the same as that of MDS and MPN [91].

More recently, mutations in cohesion complex genes (*STAG2, SMC3, SMC1A* and *RAD21*) have been discovered

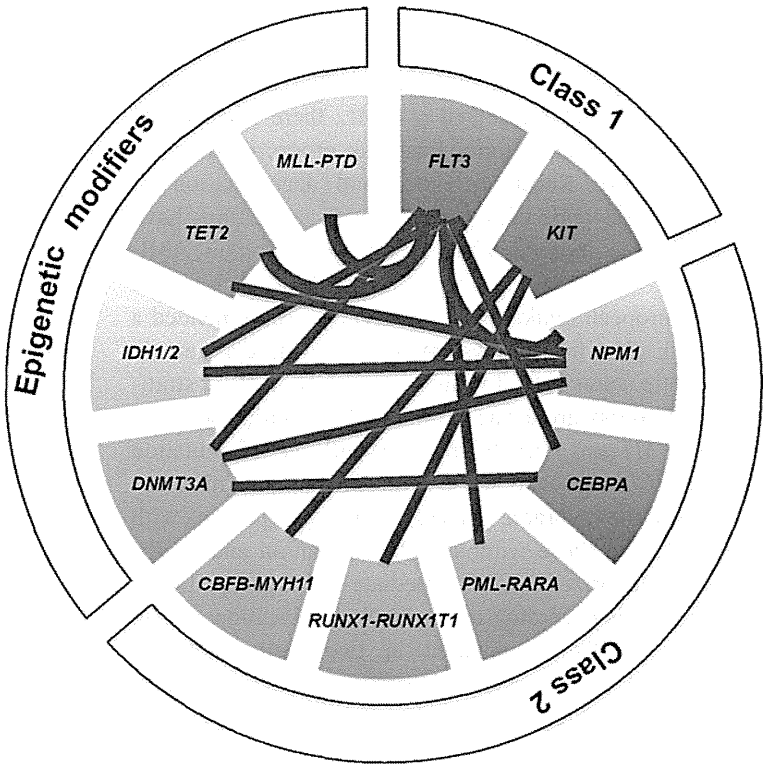

Fig. 2 Interaction of genetic alterations in AML frequently identified overlap mutations are shown. Mutations in epigenetic modifiers are overlapped both with class I and class II mutations

[40]. These mutations are reportedly mutually exclusive, indicating the redundant potential for leukemogenesis. Although mutations in cohesion complex genes were so far not identified in AML with *PML-RARA*, *CBFB-MYH11* and *BCR-ABL1*, their overlap mutations with class I, class II and epigenetic modifier mutations were identified. At present, it is unclear as to how these mutations are involved in the pathogenesis of AML, while their unique overlap pattern indicates the novel important pathway of leukemogenesis.

Several patterns of overlap mutations have been, to date, apparent as described above. However, the order of acquisition and stability of each mutation during the disease status is not fully elucidated. It has been well accepted that class II and epigenetic modifier mutations in stem/progenitor cells, which confer an advantage in self-renewal of leukemia progenitors, are earlier events than class I mutation during the development of AML. However, the interaction of class II and epigenetic modifier mutations both at the initiation and progression stages of leukemia remains unclear. Comparable analyses of mutation status between the diagnosis and the relapse of AML revealed the loss of class II mutations, such as *NPM1* and *CEBPA* mutations, at the relapse. In contrast, epigenetic modifier mutations are stably identified during the disease progression of AML, while they are frequently acquired at the later stage of MPN, suggesting their multiple oncogenic potency not only for the initiation, but also for progression in myeloid malignancies [22–24].

Background mutation

It is notable that several background mutations occur in normal hematopoietic cells [40]. Whole-genome analysis using normal hematopoietic cells revealed that the mutated gene number is lowest in the cord blood and increased according to the aging, irrespective of synonymous or non-synonymous mutation. These results suggest that background mutations, which are randomly acquired in hematopoietic cells with age and stress, might be involved in the initiation of AML development. However, these results have raised further questions regarding how background mutations affect the acquisition of driver mutations and whether driver and background mutations cooperatively induce the clonal expansion. Furthermore, it is interesting that the number of background mutations is almost the same between AML with *PML-RARA* and normal karyotype, suggesting that most of the mutations found in AML are age-related background and leukemia-associated passenger mutations. In cytogenetically normal (CN)-AML, several kinds of driver mutations are involved in the initiation of AML, while those are not always stable during the progression of AML, and the number of background mutations is the same regardless the type of initiation driver mutations [22–24, 40, 92, 93]. These findings, therefore, suggest that pathological roles of back ground mutations might be different among the types of following driver mutations.

Prognostic relevance of genetic mutations in AML

Although prognostic risk classification based on cytogenetics has been well accepted in AML, there is a clinical heterogeneity of the intermediate risk group, particularly CN-AML [94]. Recent advance and accumulation of prognostic relevance of recurrent genetic alterations have made possible more detailed risk stratification in AML patients.

The European LeukemiaNet (ELN) has recommended a novel risk classification system based on the cytogenetic and genetic status [95]. In this system, CN-AML is stratified into two risk groups according to the mutation status of *FLT3*, *NPM1* and *CEBPA*: patients with *NPM1* mutation but not *FLT3*-ITD and those with *CEBPA* mutation are included in the favorable risk (FR) group, and patients with *FLT3*-ITD and those without *NPM1* mutation and *FLT3*-ITD are categorized into intermediate-I risk group. Long-term prognosis according to the ELN classification system was retrospectively evaluated in two well-established cohorts, and both analyses demonstrated that the ELN system is useful for further risk stratification of younger adult patients with CN-AML. However, further studies are required to confirm prognostic relevance of epigenetic modifier mutations. In the patients who were treated on the Cancer and Leukemia Group B (CALGB) trials, *TET2* mutation was identified to be a poor prognostic factor in the patients who were classified in the FR group of the ELN system [47]. In the patients who were treated on the Eastern Cooperative Oncology Group (ECOG) trials, only co-occurring *NPM1* and *IDH1/2* mutations were identified to be the favorable factor for the long-term prognosis [50]. Although both analyses demonstrated possible prognostic implication of *TET2* and *IDH1/2* genes in the FR group, it was not evident in the patients registered to Munich Leukemia Laboratory in Germany [88]. Some groups reported adverse effect of *DNMT3A, MLL* and *ASXL1* mutations on long-term prognosis in AML patients, their prognostic relevance is still controversial.

Table 2 shows prognostic relevance of relatively established genetic status in AML. However, therapeutic interventions based on the genetic status should be carefully conducted, because no prospective study confirms the prognostic risk of each mutation. In the future, validation study and meta-analysis should be conducted.

Table 2 Prognostic relevance of mutation status

Associated with good prognosis	Associated with poor prognosis
NPM1-Mt/FLT3-ITD negative	FLT3-ITD
CEBPA double mutation	TP53 mutation
NPM1-Mt (for achieving CR)	TET2 mutation
IDH2 mutation	ASXL1 mutation
	PHF6 mutation

Conclusion

Comprehensive genomic analyses using the next-generation sequencer have revealed a lot of mutations in AML. Although functional and prognostic implications of each mutation are not fully elucidated, novel cooperative effects of mutations are speculated. Particularly, it is important to clarify the interactions of epigenetic modifier mutations with class I and/or class II mutations for understanding the pathogenesis and novel therapeutic targets of AML. To understand the entire picture of molecular pathogenesis in AML, gene rearrangement, gene copy number, DNA methylation and expression profiles are needed to be analyzed with gene mutations. Furthermore, it is also important to validate which mutations should be included for the prognostic stratification system. Since newly identified mutations are recurrently, but infrequently identified in AML, very large-scale prospective studies are required to confirm their prognostic implications. Finally, it is expected that genome research promotes the development of new treatment.

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Favorable outcome of patients who have 13q deletion: a suggestion for revision of the WHO 'MDS-U' designation

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ABSTRACT

To characterize bone marrow failure with del(13q), we reviewed clinical records of 22 bone marrow failure patients possessing del(13q) alone or del(13q) plus other abnormalities. All del(13q) patients were diagnosed with myelodysplastic syndrome-unclassified due to the absence of apparent dysplasia. Elevated glycosylphosphatidylinositol-anchored protein-deficient blood cell percentages were detected in all 16 with del(13q) alone and 3 of 6 (50%) patients with del(13q) plus other abnormalities. All 14 patients with del(13q) alone and 2 of 5 (40%) patients with del(13q) plus other abnormalities responded to immunosuppressive therapy with 10-year overall survival rates of 83% and 67%, respectively. Only 2 patients who had abnormalities in addition to the del(13q) abnormality developed acute myeloid leukemia. Given that myelodysplastic syndrome-unclassified with del(13q) is a benign bone marrow failure subset characterized by good response to immunosuppressive therapy and a high prevalence of increased glycosylphosphatidylinositol-anchored protein-deficient cells, del(13q) should not be considered an intermediate-risk chromosomal abnormality.

Key words: glycosylphosphatidylinositol-anchored protein-deficient, cells, bone marrow failure, 13q deletion, immunosuppressive therapy.

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Introduction

Numerical karyotypic abnormalities such as -7/del(7q) and del(13q) are occasionally seen in patients with bone marrow (BM) failure who do not exhibit typical signs of myelodysplasia. The 2008 World Health Organization (WHO) criteria defined this subset of BM failure as myelodysplastic syndrome-unclassified (MDS-U) because patient progression to leukemia was still possible. However, no large patient study has been conducted to explore an association between del(13q) and pre-leukemia.[1] Several anecdotal reports have shown that BM failure patients with del(13q) responded to immunosuppressive therapy (IST) and had a favorable prognosis.[2,3] However, the incidence of BM failure with del(13q) and its relationship with immune pathophysiology of BM failure remain unclear.

Several studies have identified the presence of small populations of glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) blood cells as a significant factor predicting a good response to IST in patients with aplastic anemia (AA) and low-risk myelodysplastic syndromes (MDS).[4,5] Immune mechanisms are, therefore, thought to be involved in the increase in the GPI-AP$^-$ cells in this type of BM failure, though the exact mechanisms responsible for the increase in the GPI-AP$^-$cells remain unknown. Given that BM failure with del(13q) is likely to respond to IST, this type of BM failure may be associated with the presence of small populations of GPI-AP$^-$ cells. It is essential to precisely characterize BM failure with del(13q) because the present WHO definition of an intermediate-risk abnormality may lead to inappropriate treatment of potentially benign BM failure with hypomethylating agents or allogeneic stem cell transplants from unrelated donors. To address this issue, the present study analyzed clinical and genetic features of 22 BM failure patients possessing del(13q) by comparing them to BM failure patients with a normal karyotype.

Design and Methods

Study subjects

Clinical records were analyzed for 1,228 BM failure patients: 733 with aplastic anemia (AA), 495 with low-risk MDS, including 286 with refractory cytopenia with unilineage dysplasia (RCUD), 149 with

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refractory cytopenia with multilineage dysplasia (RCMD) and 60 with MDS-U, whose blood samples were sent to our laboratory between May 1999 and July 2010 for screening of GPI-AP$^-$ granulocytes and erythrocytes. BM smear slides were reviewed by 2 independent hematologists. BM cellularity was defined as the percentage of BM volume occupied by hematopoietic cells in the trephine biopsy specimens. Hypocellular marrow was defined as less than 30% cellularity in patients under the age of 70 years, or less than 20% cellularity in patients 70 years and over.[6] Chromosomal analysis was performed and described according to the International System for Human Cytogenetic Nomenclature (ISCN).[7] Responses to IST were defined according to the established criteria.[8] The ethics committee of Kanazawa University Graduate School of Medical Science approved the study protocol, and all patients provided their informed consent prior to sampling.

Monoclonal antibodies

Monoclonal antibodies (mAbs) used for flow cytometry are shown on the *Online Supplementary Table S1*.

Flow cytometry for detecting GPI-AP$^-$ cells

All blood samples were analyzed within 24 h of collection to avoid false positive results due to cell damage. Staining with each mAb was performed according to the lyse-stain protocol as previously described.[5,9] The presence of CD55$^-$ CD59$^-$ glycophorin A$^+$ erythrocytes at the level of 0.005% and over and/or CD55$^-$ CD59$^-$ CD11b$^+$ granulocytes at the level of 0.003% or over was defined as an abnormal increase ('positive') based on the results obtained from 183 healthy individuals.[10] With careful handling of samples and elaborate gating strategies, cut-off values can be lowered to these levels without producing false positive results.[10-12]

Cell sorting and FISH analysis

GPI-AP$^+$ and GPI-AP$^-$ granulocytes from 2 patients with del(13q) (unique patient numbers (UPNs) 3 and 7) were sorted using a FACSAria III cell sorter (BD Bioscience, Franklin Lakes, NJ, USA) and subjected to fluorescence *in situ* hybridization (FISH) analysis using a D13S319-specific probe (Vysis, Voisins-le-Bretonneux, France) as previously described.[13]

Genome analysis of deleted region in patients with del(13q)

Genomic DNA was isolated from peripheral blood cells of 7 patients with del(13q) (UPNs 1, 3, 4, 5, 7, 8 and 22) and subjected to SNP array-based genome-wide analysis of genetic alterations using GeneChip® 250K arrays (Affymetrix, Santa Clara, California, USA) according to the manufacturer's protocol. Genomic and allele-specific copy numbers were calculated using Copy Analyser for GeneChip® (CNAG) software as previously described.[14,15]

Statistical analysis

Prevalence of increased GPI-AP$^-$ cells among different patient populations was compared using the χ^2 test. Time-to-event variables were analyzed using the Kaplan-Meier method, and groups were compared with the log rank test. Two-sided P values were calculated and $P<0.05$ was considered statistically significant. All statistical analyses were performed using the JMP software program version 8.0 (SAS Institute, Cary, NC, USA).

Results and Discussion

Of the 1,228 patients with BM failure, 22 possessed del(13q) (1.8%) that were demonstrated by G-banding; their clinical features are summarized in Table 1. Sixteen patients had only the del(13q) abnormality (which we define as 13q^{-alone}) while the remaining 6 patients had other abnormalities, which we define as 13q^{+other}. Of these 6, 2 had -Y, one had -20, one had del(7q), one had +8, and one had +mar in addition to the del(13q) abnormality. The presence of the del(13q) clone was confirmed by FISH when the number of del(13q) revealed by the G-banding method was less than or equal to two. Median age was 64.5 years old, and BM was hypocellular in 16 patients (12 with 13q^{-alone} and 4 with 13q^{+other}), normocellular in 4 (2 with 13q^{-alone} and 2 with 13q^{+other}), not evaluable in 2 with 13q^{-alone}. All patients with del(13q) were diagnosed with MDS-U due to the absence of significant dysplasia that would fullfill the criteria for MDS as defined by the 2008 WHO classification. All patients were classified as Int-1 according to the International Prognostic Scoring System (IPSS), except for UPN17 who had an IPSS score of 1.5 (Int-2).

As shown in Table 1, GPI-AP$^-$ cells that accounted for from 0.006% to 12.342% (median 0.137%) of granulocytes were detected in all 16 13q^{-alone} patients. FISH analysis of sorted GPI-AP$^-$ and GPI-AP$^+$ granulocytes revealed that del(13q) cells were derived from non-*PIGA* mutant hematopoietic stem cells (HSCs) (Figure 1A). On the other hand, the prevalence of elevated GPI-AP$^-$ cell percentages in 13q^{+other} patients and those with a normal karyotype (637 patients with AA and 300 with MDS) was 50% (3 of 6) and 43% (405 of 937), respectively ($P<0.001$).

Fourteen 13q^{-alone} patients were treated with cyclosporine (CsA) alone,[6] CsA and antithymocyte globulin (ATG)[6] or CsA and anabolic steroids;[2] all achieved either a hematologic improvement in two or three lineages or complete remission (CR), while the response rate to IST in 13q^{+other} patients was 40%. No case was IST-dependent, and response was durable after the cessation of the treatment after patients achieved CR. The clinical course of one patient (UPN 4) who responded to CsA alone and entered CR, despite the fact that G-banding of BM cells showed all 20 dividing cells to be del(13q), has been previously reported.[16] Ninety-six AA patients with the normal karyotype were treated with CsA and ATG (n=47) or CsA±anabolic steroids (n=49). Seventy-eight percent of AA patients responded to IST. Among 19 MDS patients (RCUD, n=14; RCMD, n=5) with a normal karyotype who have been treated with ATG plus CsA (n=3) or CsA with or without anabolic steroids (n=16), 63% responded to IST.

None of the 17 13q^{-alone} patients progressed to advanced MDS or acute myeloid leukemia (AML) during the follow-up period of 3-108 months (median 52 months), while 2 of 6 13q^{+other} patients (one with -20, one with del(7q)) developed AML. The 10-year overall survival rates of patients with 13q^{-alone}, patients with 13q^{+other}, AA patients with a normal karyotype and MDS (RCUD, n=38; RCMD, n=20; MDS-U, n=8) patients with a normal karyotype were 83%, 67%, 85% and 57%, respectively ($P=0.0003$, log rank test on 3 degrees of freedom) (Figure 1B). The 10-year overall survival rates of AA patients with a normal karyotype with and without increased GPI-AP$^-$ cells and MDS (38 with RCUD, 20 with RCMD and 8 with MDS-U) patients with a normal karyotype with and without increased GPI-AP$^-$ cells were 85%, 84%, 66% and 55%, respectively ($P=0.0011$, log rank test on 4 degrees of freedom) (Figure 1C). The percentage of del(13q) clones revealed by G-banding increased in 5 patients and decreased in 3 after successful IST. (*Online Supplementary*

Figure S1) No patient developed clinical features of paroxysmal nocturnal hemoglobinuria (PNH).

SNP array analysis of peripheral blood cells from 7 $13q^{-alone}$ and $13q^{-+other}$ patients indicated the region from 13q13.3 to 13q14.3 to be commonly deleted (Figure 1D).

The current retrospective study with a large number of BM failure patients revealed distinctive clinical features of BM failure with del(13q) abnormalities. The 1.8% incidence of del(13q) patients was comparable to that of a recent study (1.9%) based on 2,072 patients with MDS,[17] for which detailed diagnoses of patients with del(13q) were not provided. All del(13q) patients in our study were classified as MDS-U due to the absence of significant dysplasia. We have previously reported that response to IST was remarkably high in 9 patients with del(13q). The present study, which used a different patient cohort, confirmed our previous finding.[2] Between these 22 patients and the 9 patients that we reported in 2002, only 2 developed AML and 22 responded to IST. The overall and leukemia-free survival spans of del(13q) patients treated with IST were as long as AA patients with normal karyotypes treated with IST. These findings suggest that the del(13q) clone in BM failure patients represents the presence of immune pathophysiology rather than clonal disorder associated with AML risk.

Table 1. Clinical features of bone marrow failure patients with del(13q) alone (patients 1-16) or del(13q) plus other abnormalities (patients 17-22).

UPN	Age (years)	Sex	Months from diagnosis to sampling	Dysplasia	Cellularity	Cytogenetics	% of del(13q) cells	Break point	% GPI(-) granulocytes	% GPI(-) RBCs	Previous therapy	Treatment	Response	Outcome	AML transformation	LFS (months)
1	64	F	54	None	hypo	46,XX,del 4/20 (13)(q?)	20	13q(?)	0.042	0.015	No	CsA+AS	HI-2	alive	No	67+
2	42	M	0	None	hypo	46,XY,del(13)(q12q14) 1/20	5	13(q12q14)	3.511	0.562	No	CsA	CR	alive	No	79+
3	47	F	0	None	hypo	46,XX, del(13)(q?) 2/20	10	13q(?)	2.101	0.601	No	ATG+CsA	HI-3	alive	No	24+
4	50	F	4	Erythroid	hypo	46,XX,del(13)(q12q22) 20/20	100	13(q12q22)	0.111	0.013	No	CsA	CR	alive	No	44+
5	65	F	5	None	hypo	46,XX,del(13)(q12q14) 3/20	15	13(q12q14)	0.009	0.008	No	ATG+CsA	CR	alive	No	43+
6	21	M	1	None	hypo	46,XY, del(13)(q?) 6/20	30	13q(?)	0.038	0.003	No	ATG+CsA	HI-3	alive	No	15+
7	52	M	1	Erythroid	NE	46,XY, del(13)(q?) 19/20	95	13q(?)	12.342	0.524	PSL	CsA	HI-3	alive	No	3+
8	87	F	1	None	normo	46,XX,del(13)(q12q22) 9/20	45	13(q12q22)	0.37	0.095	No	CsA	HI-3	alive	No	15+
9	63	F	16	None	hypo	46,XX,del(13)(q12q14) del(13)(q21q31) 5/20	25	13(q12q14), 13(q21q31)	0.006	0.665	PSL	ATG+CsA	HI-3	alive	No	29+
10	74	F	3	None	hypo	46,XX, del(13)(q12q14) 7/13	54	13(q12q14)	0.504	N/A	No	ATG+CsA	HI-3	death (cancer)	No	38
11	54	F	0	None	hypo	46,XX,del(13)(q14q22) 40/40	100	13(q14q22)	0.125	0.008	No	Allo-BMT	NE	alive	No	74+
12	53	M	43	None	hypo	46,XY,del(13)(q14.3)	14	13q14.3	0.281	0.539	No	ATG+CsA	HI-3	alive	No	108+
13	85	M	1	None	hypo	46,XY,del(13)(q?) 2/20	10	13q(?)	0.031	0.01	No	No treatment	NE	death	No	10
14	77	F	3	Erythroid	NE	46,XX,del(13)(q?) 8/20	40	13q(?)	3.125	1.65	No	CsA	CR	alive	No	45+
15	56	M	1	Erythroid	normo	46,XX,del(13)(q12q14) 6/20	30	13(q12q14)	0.069	0.036	No	CsA	HI-2	alive	No	24+
16	74	M	37	None	hypo	46,XY,del(13)(q?) 7/20, 47,X,+Y 7/20	35	13q(?)	0.171	0.441	No	CsA+AS	HI-2	alive	No	52+
17	69	M	1	None	hypo	46,XY,del(7)(q22), del(13)(q12q14) 3/20	15	13(q12q14)	0	0	No	CsA+AS	NR	death	Yes	8
18	68	F	1	None	normo	45,XX,del(13)(q12q22),-20 2/20	10	13(q12q22)	0	0	No	VitK	NE	death	Yes	7
19	75	M	2	None	hypo	45,X,-Y.del(13)(q?) 2/20	10	13q(?)	0	0.003	PSL	CsA	NR	alive	No	71+
20	81	M	17	None	hypo	47,XY,+8,del(13)(q?) 19/20	95	13q(?)	6.851	0.272	No	CsA	NE	alive	No	67+
21	57	F	122	Erythroid	normo	46,XX,del(13),+mar 10/20	50	del(13)	0.522	1.075	AS	CsA+AS	HI-3	alive	No	146+
22	66	M	1	Erythroid	hypo	45,X,-Y,del(13)(q12q14) 15/20	75	13(q12q14)	0.149	0.209	No	CsA	HI-2	alive	No	11+
Median	**65**						**30**		**0.137**	**0.095**						

UPN: unique patient number; M: male; F: female; normo: normocellular marrow; hypo: hypocellular marrow; GPI-AP-granulocytes, glycosylphosphatidyl-inositol anchored protein-deficient granulocytes; GPI-AP-erythrocytes, glycosylphosphatidyl-inositol anchored protein-deficient erythrocytes; CsA: cyclosporine; ATG: antithymocyte globulin; AS: anabolicsteroid; Allo-BMT: allogeneic bone marrow transplant; VitK: vitamin K; CR: complete remission; HI-2: hematologic improvement in two lineages; HI-3: hematologic improvement in three lineages; NR: no response; NE: not evaluable; AML: acute myeloid leukemia; LFS: leukemia-free survival.

Transformation of patient 17 (UPN17) to AML could be attributed to the coexistence of del(7q), which is associated with high risk of AML evolution.[18]

The percentage change of del(13q) clone following IST varied from one patient to another (*Online Supplementary Figure S1*) in a similar way in which the percentage of GPI-AP$^-$ cells changed in the present study (*data not shown*), which is consistent with our previous findings regarding *PIGA* mutant HSCs.[10] Given that effective removal of immune mechanisms by IST does not consistently lower the percentage of del(13q) clone, it is speculated that preferential expansion of del(13q) clones by the immune mechanisms at the onset of BM failure[10] may lead to the escape from immunological pressure, as in the case of *PIGA*

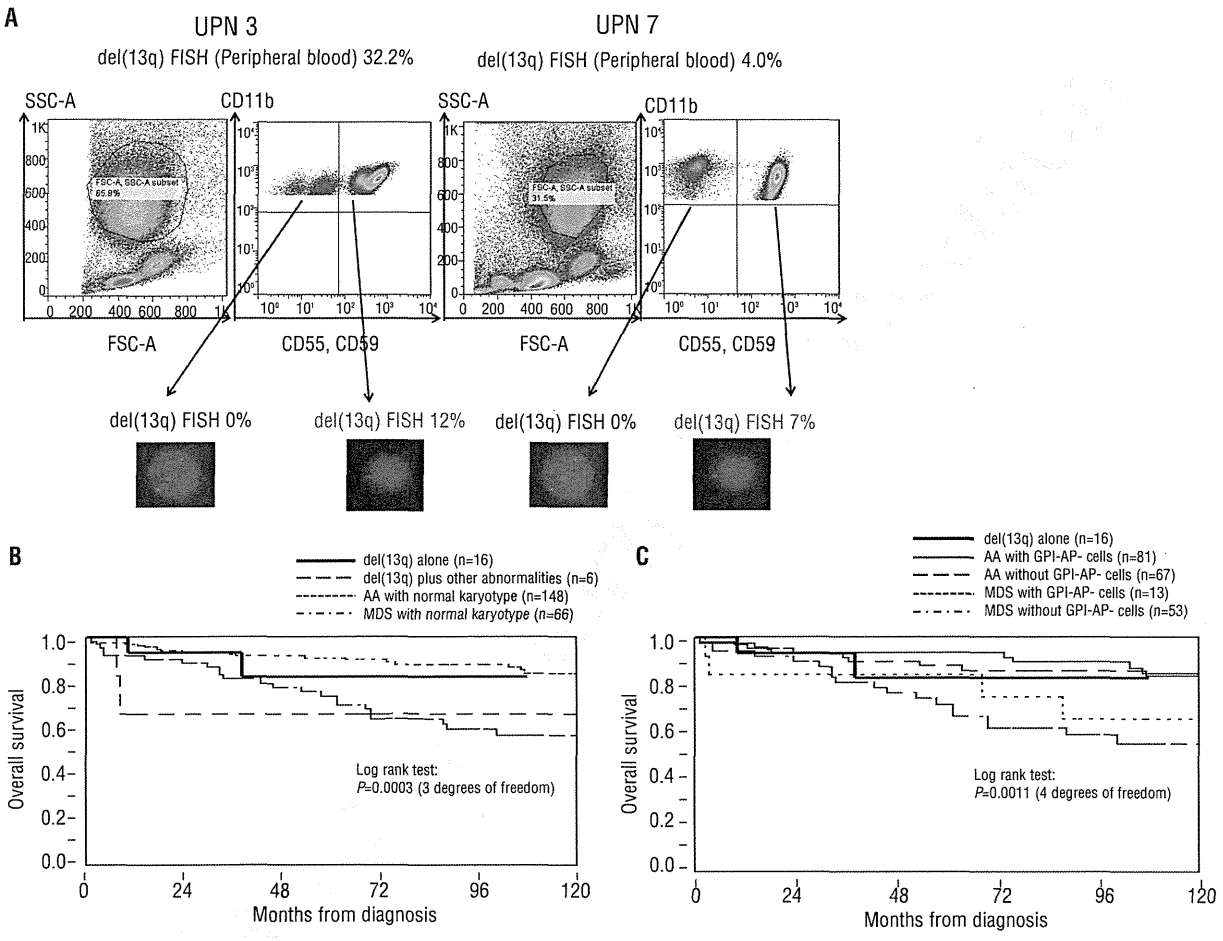

Figure 1. (A) Glycosylphosphatidylinositol-anchored protein (GPI-AP) expression in granulocytes with del(13q). GPI-AP$^-$ and GPI-AP$^+$ granulocytes were separated from peripheral blood cells of 2 patients with del(13q), one (Unique Patient Number (UPN) 3) comprised of 2.1% GPI-AP$^-$/ 32.2% del(13q) granulocytes and one (UPN 7) comprised of 12.3% GPI-AP$^-$/ 4.0% del(13q) granulocytes. Both GPI-AP$^-$ and GPI-AP$^+$ fractions were subjected to FISH analysis using a 13q probe (13q14.3); del(13q) clones were detected only in the GPI-AP$^+$ fraction of each patient (red), suggesting that del(13q) cells are derived from non-PIGA mutant hematopoietic stem cells. (B) Ten-year overall survival rates of patients with del(13q), patients with del(13q) plus other abnormalities, aplastic anemia (AA) patients with a normal karyotype and myelodysplastic syndrome (MDS) patients with a normal karyotype. All 66 MDS patients with a normal karyotype had Int-1-risk IPSS scores. (C) Ten-year overall survival rates of patients with del(13q), aplastic anemia (AA) patients with a normal karyotype with and without increased GPI-AP$^-$ cells and myelodysplastic syndrome (MDS) patients with a normal karyotype with and without increased GPI-AP$^-$ cells. (D) Deleted gene loci regions of 7 patients with del(13q), shown as bold horizontal lines for each UPN under the gene. The shaded box represents the deleted region common to patients, 13q13.3 to 13q14.3, which encodes proteins involved in cytokine signal transduction.

mutant HSCs. It is necessary to identify common mechanisms leading to preferential activation of both PIGA mutant HSCs and HSCs with del(13q) in immune-mediated BM failure to verify these hypotheses.

A possible immune pathophysiology in 13q^{-alone} patients is supported by the markedly high prevalence (100%) of elevated GPI-AP$^-$ cell levels which is linked to the escape of *PIGA* mutant HSCs from an immune system attack.[19] Because the del(13q) abnormality occurs in the GPI-AP$^+$ population, it may play a similar role to the GPI-AP$^-$ cells. SNP array analysis revealed the common deletion of a 15 Mb (13.3 to 14.3) region of 13q in 7 13q^{-alone} and 13q$^{-+other}$ patients. This segment encodes several proteins that regulate cell proliferation and the cell cycle, such as SMAD9 and RB1; both are involved in the signal transduction pathway of transforming growth factor-beta (TGF-β, an important cytokine in regulating HSC dormancy. Cytokine-mediated selection of *PIGA* mutant HSCs has been proposed as a mechanism for preferential proliferation of GPI-AP$^-$ cells,[20] but no supporting evidence has been presented. A previous study demonstrated that GPI-AP$^-$ T cells show decreased sensitivity to herpes virus entry mediator (HVEM) ligands that transmit inhibitory signals through receptors for CD160(21) and TGF-β.[22,23] The presence of del(13q) represents a unique subgroup of immune-mediated BM failure associated with an increase in the percentage of GPI-AP$^-$ cells, where del(13q) and *PIGA* mutant HSCs undergo preferential expansion, possibly due to their decreased sensitivity to cell-cycle inhibitory molecules, such as TGF-β compared to normal HSCs.

In conclusion, MDS-U with del(13q) alone is a benign BM failure syndrome characterized by a good response to IST and a markedly high prevalence of elevated GPI-AP$^-$ cell percentages. Therefore, del(13q) should be eliminated from the list of karyotypic abnormalities representing the intermediate group defined by IPSS,[24] and BM failure with del(13q) should be managed as AA.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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Individual Hematopoietic Stem Cells in Human Bone Marrow of Patients with Aplastic Anemia or Myelodysplastic Syndrome Stably Give Rise to Limited Cell Lineages

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Key Words. Differentiation • Experimental models • Fluorescence-activated cell sorting • Hemopoietic stem cells • Hematopoietic progenitors • Aplastic anemia • Bone marrow

ABSTRACT

Mutation of the phosphatidylinositol *N*-acetylglucosaminyltransferase subunit A (*PIG-A*) gene in hematopoietic stem cells (HSCs) results in the loss of glycosylphosphatidylinositol-anchored proteins (GPI-APs) on HSCs, but minimally affects their development, and thus can be used as a clonal maker of HSCs. We analyzed GPI-APs expression on six major lineage cells in a total of 574 patients with bone marrow (BM) failure in which microenvironment itself is thought to be unaffected, including aplastic anemia (AA) or myelodysplastic syndrome (MDS). GPI-APs-deficient (GPI-APs$^-$) cells were detected in 250 patients. Whereas the GPI-APs$^-$ cells were seen in all six lineages in a majority of patients who had higher proportion ([dbmtequ]3%) of GPI-APs$^-$ cells, they were detected in only limited lineages in 92.9% of cases in the lower proportion (<3%) group. In all 250 cases, the same lineages of GPI-APs$^-$ cells were detected even after 6–18-month intervals, indicating that the GPI-APs$^-$ cells reflect hematopoiesis maintained by a self-renewing HSC in most of cases. The frequency of clones with limited lineages seen in mild cases of AA was similar to that in severe cases, and clones with limited lineages were seen even in two health volunteer cases. These results strongly suggest most individual HSCs produce only restricted lineages even in a steady state. While this restriction could reflect heterogeneity in the developmental potential of HSCs, we propose an alternative model in which the BM microenvironment is mosaic in supporting commitment of progenitors toward distinct lineages. Our computer simulation based on this model successfully recapitulated the observed clinical data. STEM CELLS *2013;31:536–546*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

To sustain hematopoiesis, hematopoietic stem cells (HSCs) must, on the one hand, replenish themselves by self-renewal and on the other hand produce differentiating progenitor cells. It is also known that most HSCs remain dormant and are only rarely and randomly activated. It has been estimated that each human possesses a total of 10^4 HSCs, and that ~400 HSCs actively contribute to hematopoiesis, replicating once per year [1, 2]. However, the actual dynamics of hematopoiesis by HSCs remains uncertain. For example, it is unclear how long an individual HSC maintains hematopoiesis and whether all major lineage cells are produced from a single HSC. These issues have been difficult to address due to the lack of appropriate experimental systems, regardless of animal species.

In the case of humans, however, we were led to consider one "experiment of nature" that makes it possible to track the

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progeny of a HSC; that is, by detecting blood cells deficient in glycosylphosphatidylinositol-anchored proteins (GPI-APs) using flow cytometry. GPI-APs-deficient (GPI-APs$^-$) blood cells are rarely detectable in healthy individuals, but are a common feature in paroxysmal nocturnal hemoglobinuria (PNH) and are also frequently seen in patients with bone marrow (BM) failure such as aplastic anemia (AA) or myelodysplastic syndrome (MDS). These cells are known to be derived from HSCs with a mutation in the phosphatidylinositol *N*-acetylglucosaminyltransferase subunit A (*PIG-A*) gene. MDS cases harboring GPI-APs$^-$ blood cells are characterized by polyclonal hematopoiesis and good response to immunosuppressive therapy, and are therefore thought to be similar to AA in their pathophysiology [3].

The *PIG-A* mutant HSCs used to be thought to have greater proliferative capacity than normal HSCs because one or a few *PIG-A* mutant clones sometimes account for a large proportion of hematopoietic cells for long period in PNH patients [4]. However, several reports instead indicate that the mutant HSCs in most PNH patients have properties similar to normal HSCs: for example, no growth advantage over wild-type HSCs [5, 6], genetic stability and extremely low incidence of secondary mutations [2, 7–9], and the rarity of leukemic cell evolution of GPI-APs$^-$ cells in PNH patients [10].

We recently found that GPI-APs$^-$ cells in patients with AA and MDS frequently show various patterns in the proportion of granulocytes and erythrocytes and that the individual patterns of the two lineage combinations can persist for many years. The percentage of GPI-APs$^-$ cells in some patients remained almost the same over 10 years even when the percentages were less than 1% [11]. In such stable cases, it is quite likely that GPI-APs$^-$ peripheral blood (PB) cells are produced from HSC(s), and that the GPI-APs$^-$ HSC itself as well as its surrounding environment is largely normal, at least during the observation period. Indeed, BM environment itself in the patients to support hematopoiesis is thought to be largely normal, since these cases are thought to be caused by immune reaction against blood cells. It is also highly probable that the whole GPI-APs$^-$ cells in each patient represent a clone, originating from a single HSC in which *PIG-A* mutation occurred, since it is statistically rare that the *PIG-A* mutation occurs twice in one patient. Although it is unclear that such a clone is maintained by a single HSC or multiple descendent HSCs, it can be said that the kinetics of GPI-APs$^-$ cells during stable period reflect the regular hematopoietic events originally initiated by a single HSC. We then thought that it could be very much informative if we extend our analysis to cover various hematopoietic lineages in addition to erythrocytes and granulocytes. We therefore determined the proportion of GPI-APs$^-$ cells in six major lineages, namely granulocytes (G), monocytes (M), erythrocytes (E), T cells (T), Natural Killer cells (NK), and B cells (B), in PB cells from BM failure patients using a highly sensitivity flow cytometric analysis [12].

Materials and Methods

Patients and Healthy Volunteers

The PB of 574 patients with various types of cytopenias was examined for the presence of GPI-APs$^-$ cells using high sensitivity flow cytometry. Their diagnoses included AA in 354 (39 with very severe, 92 with severe, and 223 with nonsevere AA [13]), MDS-refractory anemia (RA) defined by the french-american-british (FAB) classification [14] in 207, and classic PNH in 13. The male to female ratio was 1:1.2 (261:313) and the median age was 57 years (range: 1–95 years). PB samples from 192 healthy individuals were also examined for the presence of GPI-APs$^-$ cells in all lineages of cells. All patients and healthy individuals including next of kin on the behalf of minors/children participants in our study provided their informed written consent before sampling. This study protocol was approved by the ethics committee of Kanazawa University Graduate School of Medical Science.

Monoclonal Antibodies

Monoclonal antibodies (mAbs) used for multicolor flow cytometry were anti-CD59 labeled with FITC (P282E, IgG2a; Beckman Coulter, Miami, FL, http://www.labome.com/product/Beckman-Coulter-Inc/IM3457U.html), anti-CD55 labeled with FITC (IA10, IgG2a; BD Pharmingen,http://www.bdbiosciences.com/external_files/pm/doc/tds/human/live/web_enabled/33574X_555693.pdf), anti-CD48 labeled with FITC (J4-57, IgG1; Beckman Coulter, http://www.bionity.com/en/antibodies/beckman-IM1837U/cd48-anti-human-klon-j4-57.html), anti-CD33 labeled with APC (D3HL60.251, IgG1; Beckman Coulter, http://www.bionity.com/en/antibodies/beckman-A70200/cd33-anti-human-klon-d3hl60-251.html), anti-CD19 labeled with APC-Cy7 (SJ25C1, IgG1; BD Pharmingen, http://www.bdbiosciences.com/external_files/pm/doc/tds/human/live/web_enabled/557791.pdf), anti-CD335 labeled with Phycoerythrin (BAB281, IgG1; Beckman Coulter, http://www.bc-cytometry.com/DataSheetPDF/IM3711_D.S.pdf), anti-CD3 labeled with PerCPCy5.5 (SK7, IgG1; BD Pharmingen, http://www.bdbiosciences.com/documents/BD_PerCP-Cy5.5_and_PerCP.pdf), anti-CD11b/Mac-1 labeled with PE (ICRF44, IgG1; BD Pharmingen, http://www4.bdj.co.jp/ecat/txDetailedTable.jsp?size=20&item=746256&form=formNavigator&action=LIST_PAGE&pageItem=45), and anti-glycophorin A labeled with PE (JC159, IgG1; Dako, Carpenteria, CA, http://www.dako.com/us/index/flow_cytometry_catalog_us.pdf).

Flow Cytometry for Detecting GPI-APs$^-$ Cells

Six lineages of blood cells including granulocytes, erythrocytes, monocytes, T cells, B cells, and NK cells were subjected to high sensitivity flow cytometry for detecting small populations of GPI-APs$^-$ cells. All blood samples were analyzed within 24 hours to avoid false-positive results due to cell damage. The staining with the each mAb in this study was performed according to the well-established lyse-stain protocol, previously described in detail [12, 15]. Briefly, 3–5 mL of heparinized blood was drawn from the patients and healthy individuals. Erythrocytes were lysed in a lysis buffer (Roche Applied Science, Nagoya, Japan, http://roche-biochem.jp/catalog/index.php/product_3.6.6.4.42.1.html) containing NH_4Cl 8.26 g/L, $KHCO_3$ 1.0 g/L, and EDTA·E_4Na 0.037 g/L to detect GPI-APs$^-$ leukocytes. After washing with saline, 50 μL of the leukocyte suspension was incubated with FITC-labeled anti-CD55 and anti-CD59 mAbs for granulocytes or FITC-labeled anti-CD48 and anti-CD59 mAbs for monocytes, T cells, B cells, and NK cells in combination with mAbs specific for lineage markers including PE-labeled CD11b for granulocytes, APC-labeled CD33 for monocytes, PerCP-Cy5.5-labeled CD3 for T cells, APC-Cy7-labeled CD19 for B cells, and PE-labeled CD335 for NK cells. Fresh blood was diluted to 3% in phosphate-buffered saline (PBS), and then 50 μL was incubated with PE-labeled anti-glycophorin A and FITC-labeled anti-CD55 and anti-CD59 mAbs on ice for 30 minutes to detect GPI-APs$^-$ erythrocytes. Three-step gating excluded debris and immature granulocytes that are frequently found in samples from patients with MDS. Step 1 involved the gating of granulocyte, lymphocyte, or monocyte populations from the Forward Scatter (FSC)-Side Scatter (SSC) scattergrams (R1). Step 2 involved the gating of the lineage marker^{bright} population on the lineage marker-SSC scattergram to exclude the lineage marker^{dim} cells that are features of either damaged cells or immature cells. Step 3 was the gating of R1 × R2 and the analysis of 10^6 cells on R1 × R2 scattergrams. The following cut-off values that had been determined by our

previous studies based on 183 healthy individuals were used; the presence of ≥0.005% $CD55^-CD59^-$ glycophorin A^+ erythrocytes, ≥0.003% $CD55^-CD59^-CD11b^+$ granulocytes, and ≥0.01% $CD48^-CD59^-CD33^+$ monocytes, $CD48^-CD59^-CD3^+$ T cells, $CD48^-CD59^-CD19^+$ B cells, and $CD48^-CD59^-CD335^+$ NK cells [12, 16]. When GPI-APs$^-$ cells were detected in only one lineage of cells or the percentages of GPI-APs$^-$ cells were less than 0.01%, then additional samples were tested, and the patients were judged to be positive for increased GPI-APs$^-$ (PNH^+) when the analysis results of the first and second samples were identical. Data acquisition was performed immediately after the sample preparation using a FACSCanto II instrument (Beckton Dickinson) and the data were analyzed using the FACSDiva software program and the percentage of each population was calculated by FlowJo software 7.6.1 (Tree star, Inc., Ashland, OR).

Cell Sorting and *PIG-A* Gene Analysis

Freshly isolated GPI-APs$^-$ cells were separated from GPI-APs$^+$ fraction using a FACSAria II instrument (Beckton Dickinson). More than 95% of the sorted cells were GPI-APs deficient. An analysis of the *PIG-A* gene mutation was performed as described previously [17]. Briefly, the coding regions of *PIG-A* were amplified by nested or seminested PCR using 12 primer sets, and six ligation reactions were used to transform competent *Escherichia coli* JM109 cells (Nippon Gene, Japan, http://www.nippongene.com/pages/products/genetransfer/ecos/index.html). Five clones were selected randomly from each group of transfectants and subjected to sequencing with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, San Diego, CA,http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042772.pdf) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Simulation of the Blood Cell Production by HSCs

Migration, division, lineage determination, and death of clones of a HSC in BM environment are simulated with a lattice Monte Carlo method. For efficient simulation, we focused on the events on a cross-section of BM, which enabled us to perform two-dimensional simulation. The occurrence of events was managed with their transition probabilities and the simulation was executed on the basis of actual time. We used the hybrid null-event Monte Carlo algorithm [18, 19], and here outline the model description and simulation setup (also see Supporting Information Note for detailed information). A square area (30 mm × 30 mm) that is large enough to encompass some commitment planes is represented using 1,500 × 1,500 square lattices (20 μm × 20 μm each). Initially, a HSC is randomly added to a site, and then it continues to create clones once per 10 hours until there have been five divisions. Clones randomly move along the lattice with the transition probability and undergo cell division once per 8–12 hours. We assume that lineage determination occurs after the fifth division, based on the site of the clone in the mosaic-like hematopoietic environment where each site supports differentiation into M, E, B, or T-cell lineages. In addition, the lineage clone becomes a mature cell after a further five divisions under the condition that it can divide only in the sites that support differentiation into the same lineage. Otherwise, cell death results after 48-hour movement on areas that support other lineages, although all clones die 72 hours after maturation.

We also investigated the relation between clone size and the number of emerging cell lineages. In our simulation, clone size is dominated by the setting of "N times divisions for lineage determination and further N times divisions for maturing." We simply change the number N in a range of 3–12. For each N, 128 simulations starting from randomly selected initial sites of HSCs are executed for 120 simulation hours. For each simulation, the number of cell lineages is counted, and clone size is calculated as the maximum number of clones for the entire simulation period.

Results

GPI-APs$^-$ Cells Were Seen in Various Combination of Lineages of Blood Cells from BM Failure Patients

Of 574 patients with BM failure, GPI-APs$^-$ cells were detected in at least one lineage of cells of 250 patients (44%). The prevalences of increased GPI-APs$^-$ cells were 56% in AA and 19% in MDS-RA. The proportion of GPI-APs$^-$ cells among granulocytes ranged 0.003%–99.1%, with mean and median value 5.38% and 0.19%, respectively. GPI-APs$^-$ cells were rather frequently found in patients with less severe AA; the prevalences were 63% in nonsevere, 49% in severe, and 31% in very severe AA patients. The lineage combinations of GPI-APs$^-$ cells in patients possessing GPI-APs$^-$ cells (PNH^+ patients) were classified into 16 different patterns (Supporting Information Table S1). Figure 1 shows representative flow cytometric profiles of one healthy individual and four patients showing Granulocyte, Monocyte, Erythrocyte, T cell, NK cell, B cell (GMETNKB), Granulocyte, Monocyte, Erythrocyte (GME), Granulocyte, Monocyte, Erythrocyte, T cell, NK (GMETNK), or Granulocyte, Monocyte, Erythrocyte, B cell (GMEB) patterns.

Clone Size of GPI-APs$^-$ Granulocytes Correlates with the Number of Cell Lineages that Contain GPI-APs$^-$ Cells

The percentages of GPI-APs$^-$ granulocytes in each group bearing GPI-APs$^-$ cells in one to six lineages are plotted in Figure 2. There was a clear trend toward the pattern of the higher the percentage of GPI-APs$^-$ granulocytes in patients, the greater the number of GPI-APs$^-$ cell lineages.

In order to closely examine the relationship between the severity of AA and the GPI-APs$^-$ clone size, we arbitrarily classified AA patients in this study into five categories, namely stages 1, 2, and 3 (nonsevere), stage 4 (severe), and stage 5 (very severe), based on the severity of cytopenias (Supporting Information Table S2) and replotted the data (Fig. 2B). Patients with higher proportion (>1%) of GPI-APs$^-$ granulocytes were not seen in nonsevere cases, suggesting that the clone size of GPI-APs$^-$ cells correlates with severity of BM failure. The notable observation here is that the correlation between the percentage of GPI-APs$^-$ granulocytes and the number of GPI-APs$^-$ cell lineages is almost identical in all three groups. This finding may indicate that the production of limited lineage cells observed in smaller clones is not due to the possible functional failures of BM microenvironment. It seems more likely that "fewer lineages in smaller clones" instead represents events occurring in normal hematopoiesis.

Persistence of GPI-APs$^-$ Lineage Combination Over a Long Period

We then reexamined PB cells of 250 patients 6–18 months after the first examination and to our surprise, GPI-APs$^-$ cells were observed in all 250 patients. Detection of GPI-APs$^-$ cells after such a long interval indicates that the GPI-APs$^-$ cells are most likely derived from HSCs rather than from non-self-renewing progenitors. Of special interest was our finding that in all 250 cases, the same combinations of lineages were detected regardless of the interval between the first and second analysis. Flow cytometric profiles of various lineages of a case representing Granulocyte, Monocyte, Erythrocyte, T cell (GMET) type are shown in Figure 3. Figure 4 shows the proportion of GPI-APs$^-$ cells in each lineage in the first and second analysis for a total of representative 25 cases (five cases for each type).

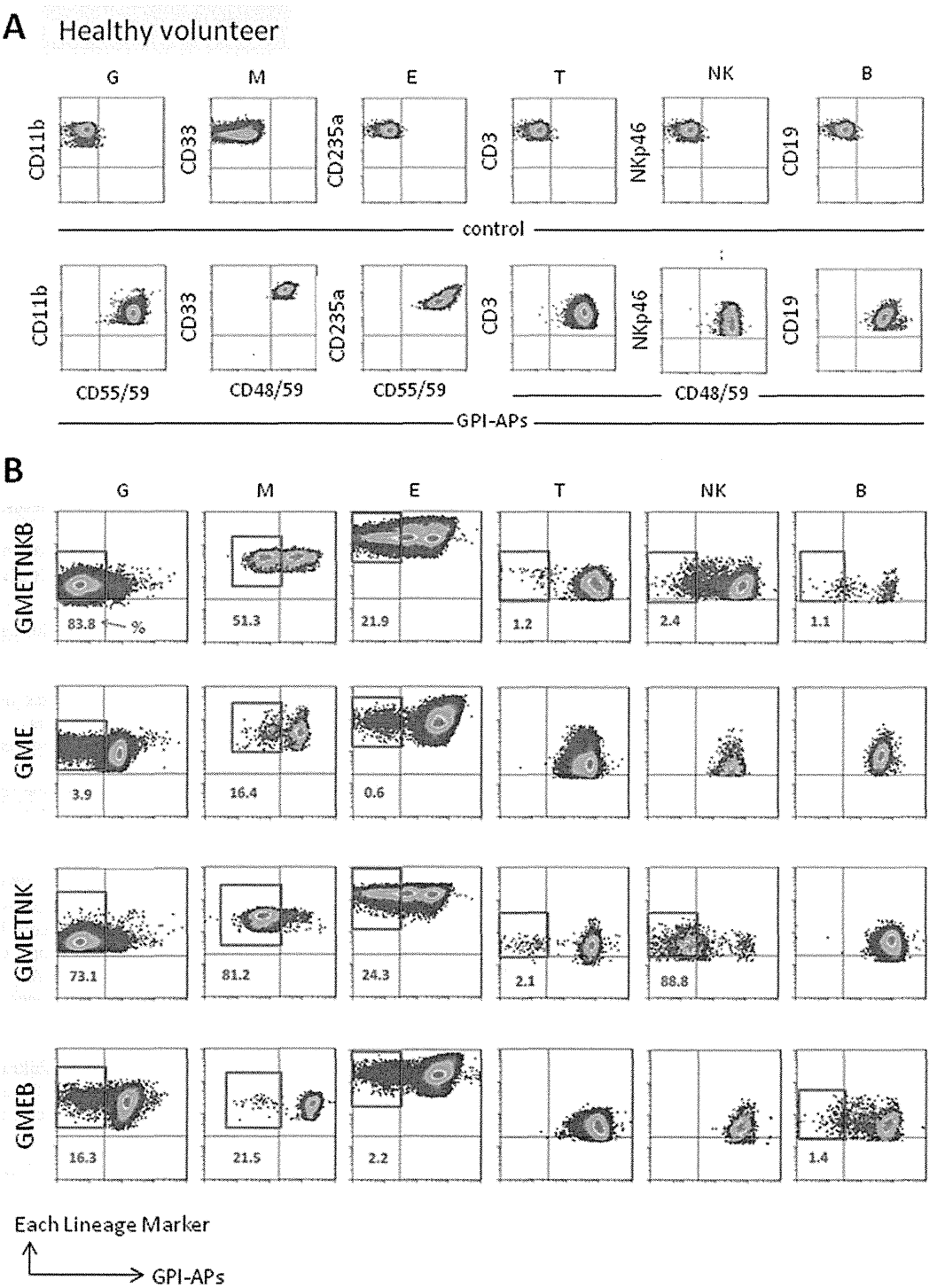

Figure 1. Detection of GPI-APs$^-$ cells in different lineages of blood cells. **(A):** One example of peripheral blood (PB) cells of a healthy individual having no GPI-APs$^-$ cells. **(B):** GPI-APs$^-$ cell combination patterns in four patients. Profiles of PB cells of patients showing GPI-APs$^-$ cells in GMETNKB, GME, GMETNK, and GMEB lineages are shown. Lineage markers used were CD11b for G, CD33 for M, glycophorin-A (CD235a) for E, CD3 for T, NKp46 for NK, and CD19 for B. G, M, E, T, NK, or B stands for granulocytes, monocytes, erythrocytes, T cells, NK cells, or B cells, respectively. Abbreviations: GPI-APs, glycosylphosphatidylinositol-anchored proteins; GME, Granulocyte, Monocyte, Erythrocyte.

PIG-A Gene Mutations in Different Lineages of Blood Cells

To confirm their clonal origin, GPI-APs$^-$ cells sorted from five patients were subjected to *PIG-A* gene analysis. The same mutation was identified in different lineages in three patients (Supporting Information Table S3). In GPI-APs$^-$ granulocytes with other lineages from Patients 16, 1, and 5 and GPI-APs$^-$ granulocytes from Patients 9 and 30, the same

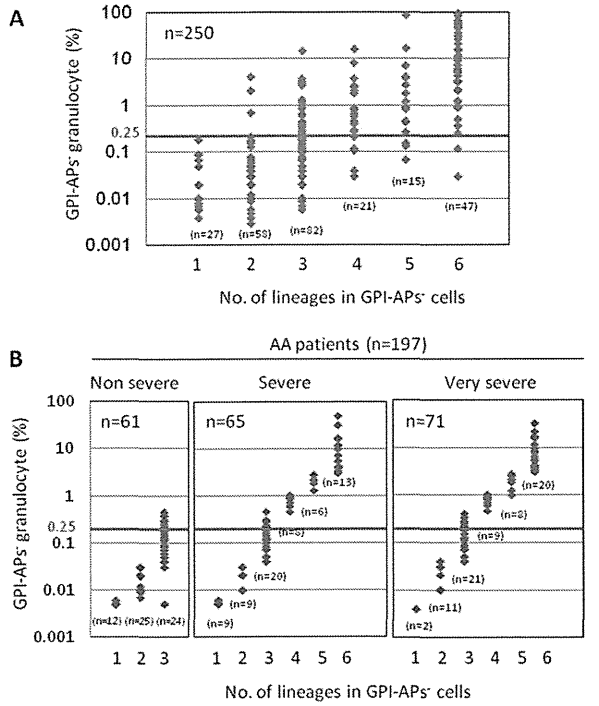

Figure 2. Correlation between the clone size of GPI-APs$^-$ granulocytes and the number of cell lineages that contain GPI-APs$^-$ cells. **(A):** The percentage of GPI-APs$^-$ granulocytes and the number of cell lineages that contain GPI-APs$^-$ cells in individual patients are plotted. A red line for 0.25% stands for the expected value for an average size of a single hematopoietic stem cell clone. **(B):** The percentage of GPI-APs$^-$ granulocytes and the number of cell lineages that contain GPI-APs$^-$ cells in AA patients, classified in three categories according to the severity of disease (stage 1 or 2, stage 3, and stage 4 or 5), are plotted. AA patients were classified into five categories based on the disease severity (stage 1 or 2, stage 3, and stage 4 or 5) (Supporting Information Table S2). Abbreviation: GPI-APs, glycosylphosphatidylinositol-anchored proteins.

mutation was found 3–7 months after the initial analyses. Therefore, it is highly probable that in most, if not all, cases the GPI-APs$^-$ cells are clonal [20]. This finding is in line with the estimation that PNH patients only rarely have more than two clones at the HSC level [8, 9].

GPI-APs$^-$ Cells Were Detected in Limited Lineages in Healthy Volunteers Over a Long Period

Dormant HSCs with *PIG-A* mutation reside in the BM and can be activated albeit uncommonly [17]. If this hypothesis is tenable, small populations of GPI-APs$^-$ cells may be detectable in some healthy individuals. We then examined PB of 192 healthy volunteers for the presence of GPI-APs$^-$ cells. Notably, two were found to bear detectable levels of GPI-APs$^-$ cells, and in both of them GPI-APs$^-$ cells were detected in limited lineages, representing GME and GE type (Fig. 5A). The two healthy cases bearing GPI-APs$^-$ cells were situated within the range of distribution of AA and MDS cases in terms of chimerism ratio versus lineage number (Fig. 5B), further supporting that findings seen in AA and MDS cases reflect normal hematopoiesis.

As described in Introduction, the number of active human HSCs at any given time is estimated to be around 400. It is therefore likely that the GPI-APs$^-$ clones with a frequency of less than 0.25% reflect hematopoiesis maintained by a single HSC. It is clear from our studies that most of these small clones contain only limited lineage cells. Even in the case of larger clones, for example, clones of 1%–3% chimerism that might be maintained more than two HSCs, the majority (81%) are non-full-lineage clones. Collectively, these results indicate that most individual human HSCs only give rise to a limited range of hematopoietic progeny.

A Model for the Hematopoietic Microenvironment to Explain the Production of Limited Cell Lineages from a HSC

A cogent explanation for this phenomenon may have important implications for normal hematopoiesis. We

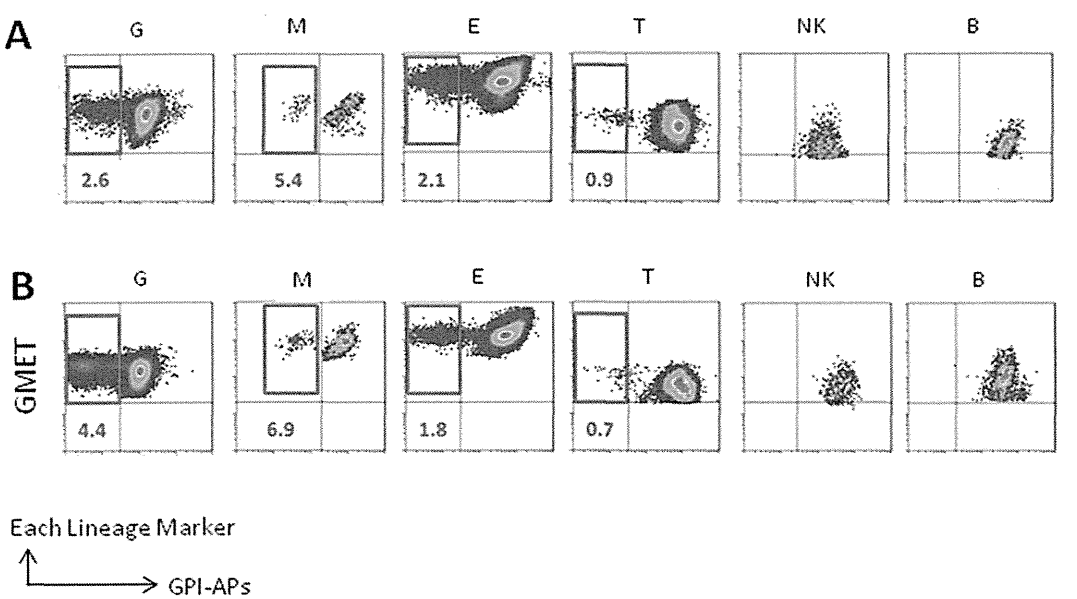

Figure 3. The lineage combination pattern of GPI-APs$^-$ cells was same at the first sampling **(A)** and after 6 months **(B)**. Abbreviation: GPI-APs, glycosylphosphatidylinositol-anchored proteins.