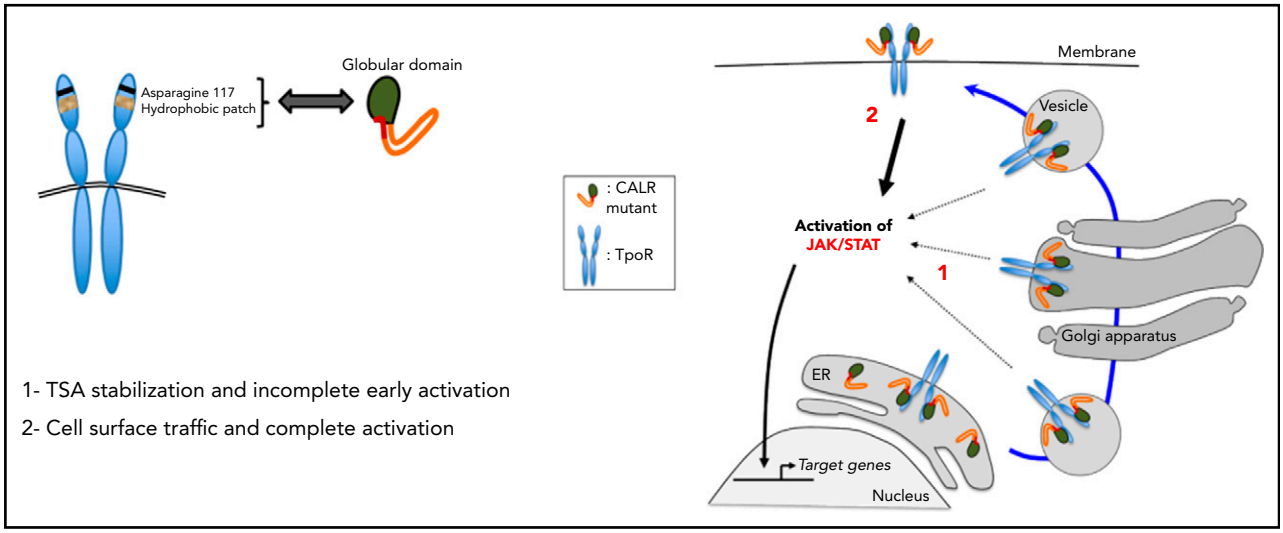

Diagram of the principle features of the mutated CALR–TpoR interaction leading to cell surface transport from the ER, TpoR activation, and signal transduction. See Figure 6E in the article by Pecquet et al that begins on page 2669.

focused on inhibiting the ER chaperone function of JAK2^{V617F} might be profitably engaged on inhibiting the chaperone function of mutated CALR. Second, the observation that the TpoR MPL^{R102P}, when expressed at the cell surface, can be stimulated by a Tpo mimetic implies that, in the future, *CALR* gene editing might be a useful strategy for correcting CAMT. Finally, the expression of a neoantigen, mutated CALR, on the surface of MPN HSCs provides a rationale for immunotherapy in *CALR* mutation–positive MPN patients.[1]

Unsurprisingly, interesting experimental observations lead to new inquiries. In this instance, such inquiries would include the mechanism for wild-type JAK2 activation by mutated CALR, how wild-type JAK2 activated by mutated CALR avoids physiologic inhibition, and the length of time that mutated CALR–TpoR complexes reside on the cell surface. Normally, Tpo-induced JAK2 activation is accompanied by upregulation of SOCS3 and LNK, which attenuate JAK2 activity but not TpoR recycling. JAK2^{V617F} paradoxically abbreviates the cell surface residence of mature TpoR by ubiquitination and blocks TpoR recycling,[10] but it remains to be determined whether mutated CALR mimics JAK2^{V617F} in this regard.

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MYELOID NEOPLASIA

Comment on Nakamura et al, page 2682

Message from the void: MRD analysis from ctDNA

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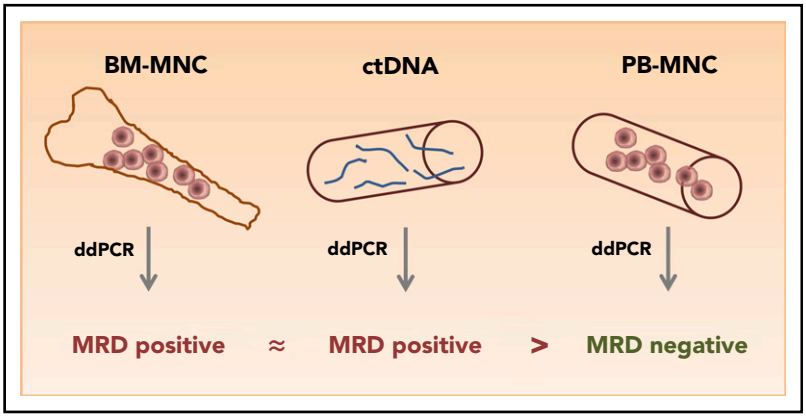
In this issue of *Blood*, Nakamura et al demonstrate that circulating tumor DNA (ctDNA) in the serum can be used for minimal residual disease (MRD) assessment in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patients after allogeneic hematopoietic cell transplantation and thus may relieve patients from frequent bone marrow punctures.[1]

Compared with solid cancers, hematologists have long benefited from easily accessible tumor sampling from blood and bone marrow. This ability cemented the dogma that bone marrow sampling from the pelvis provides a representative



MRD from ctDNA shows comparable sensitivity to bone marrow (BM) MRD in AML and MDS patients after alloSCT. MRD was measured by ddPCR using individual gene mutations as MRD markers. Some patients were MRD positive by ctDNA analysis but MRD negative in mononuclear cells (MNCs) from peripheral blood (PB), indicating a better representation of residual leukemia in ctDNA than PB-MNCs.

sample to ascertain the remission status of acute leukemias and MDS. However, AML can be exclusively located at extramedullary sites at relapse or even at diagnosis, and acute lymphoblastic leukemia may relapse predominantly in lymphoid tissues. Nakamura and colleagues have now developed a method to monitor AML in patient serum using ctDNA, providing a platform to investigate important questions, including whether AML is homogeneously or distinctly distributed in the body.[1]

Early measurement of treatment response is increasingly recognized as an important tool to predict final treatment outcome of AML patients. The European LeukemiaNet recommends refining response assessment through the measurement of residual disease (MRD).[2] However, the National Comprehensive Cancer Network AML guidelines do not recommend routine MRD analysis in clinical practice.[3] Flow cytometry and real-time polymerase chain reaction (PCR)–based approaches are currently recommended for MRD assessment,[4] with next-generation sequencing (NGS) becoming more frequently used.[5,6] Nakamura and colleagues quantified MRD in AML and MDS patients after allogeneic hematopoietic stem cell transplantation (alloSCT) by droplet digital PCR (ddPCR) with a median detection limit of 0.04%. MRD positivity predicted relapse and correlated with shorter overall survival. Interestingly, MRD analysis from ctDNA identified MRD in some patients who were MRD negative in peripheral blood and/or bone marrow, indicating that ctDNA may be more representative of residual AML in some patients.

The authors screened 53 patients for gene mutations by NGS and identified at least one mutation in 51 patients. Allele-specific PCR assays were designed for each selected mutation and validated separately. MRD was monitored in ctDNA and bone marrow cells 1 and 3 months after alloSCT in AML and MDS patients who had undergone myeloablative conditioning. Variant allele frequencies (VAFs) were highly correlated between the diagnostic ctDNA and bone marrow. MRD positivity using conventional or ctDNA technologies predicted a higher relapse rate and shorter overall survival compared with MRD-negative patients both 1 and 3 months after alloSCT. The authors conclude that ctDNA monitoring provides a convenient approach to MRD monitoring with comparable sensitivity to using bone marrow and improved sensitivity to using peripheral blood cells, especially in cytopenic patients after alloSCT (see figure). This comprehensive study provides a new set of diagnostic assays for prognostication and fuels the hope that patients will require fewer bone marrow assessments after alloSCT in the future.

What is the source of the ctDNA? Is it actively secreted or shed from dying leukemic cells to the serum? This study and previous reports have observed that higher levels of cell-free DNA are found in cancer patients than in healthy controls and that circulating tumor cells do not correlate well with ctDNA levels, supporting the active secretion hypothesis[7] and providing a possible explanation for why VAFs measured in ctDNA and bone marrow are comparable. It is surprising that 10 ng ctDNA was sufficient to detect low levels of MRD, as other DNA-based MRD assays recommend using 600 to 2000 ng DNA to achieve a minimum sensitivity of 1 leukemic cell in 100 000 normal cells. Preferential DNA secretion from tumor cells over normal cells might enrich the tumor DNA in serum and partially explain the good sensitivity from low DNA input. In contrast, there is a clear gradient of leukemic cells from bone marrow to peripheral blood, which reproducibly results in a 10-fold lower frequency of leukemic cells in peripheral blood compared with bone marrow in AML patients with low MRD. However, active DNA secretion from tumor cells remains speculative, as the mechanism by which this occurs and its regulation remains unknown.

The strong prognostic effect of MRD positivity as early as 1 month after myeloablative alloSCT demonstrated by Nakamura and colleagues is impressive. At this time point, many patients are still cytopenic, and other MRD techniques such as flow cytometry show less discriminatory potential after alloSCT compared with an assessment before alloHCT.[8] It will be interesting to see the potential of this method in other hematologic diseases like lymphoma and myeloma, which are usually less represented in peripheral blood than acute leukemias.

A potential limitation of ddPCR is the requirement for a unique assay for each nucleotide change. It is therefore most suitable for recurrent mutations in genes like *IDH1* and *IDH2* and other genes with hotspot mutations. However, ctDNA can be also examined by error-corrected NGS, so the most convenient and reproducible approach will need to be established by individual laboratories.

In summary, the current study extends our tools to sensitively monitor MRD in AML and MDS and provides the technical and conceptual framework to understand asynchronous development of leukemic clones at different sites, mechanisms of DNA release from tumor cells, and potential biologic functions of circulating tumor DNA. While the serum of leukemia patients has largely been considered free of leukemia-specific information, this void is beginning to speak to us, and its next messages are eagerly expected.

Conflict-of-interest disclosure: The authors declare no competing financial interests. ■

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PLATELETS AND THROMBOPOIESIS

Comment on Volz et al, page 2696

GPVI inhibitor as antitumor gateway drug

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In this issue of *Blood*, Volz et al establish a potential antitumor strategy by exploiting the selective requirement for platelets to maintain vascular integrity within the tumor microenvironment.[1] Their work demonstrates, for the first time, that functional inhibition of platelet-specific surface receptor glycoprotein (GP) VI, using F(ab')$_2$ fragments to avoid platelet clearance, increases intratumoral hemorrhage and concomitant tumor cell apoptosis, as well as enhanced accumulation of chemotherapeutic drugs. These effects work additively to inhibit tumor growth, achieving results similar to those achieved by platelet depletion.[2,3]

Among platelet receptors, GPVI possesses the rare property of being nonessential for hemostasis, but its loss or blockade prevents arterial thrombosis, making GPVI an attractive target. Indeed, Revacept, a soluble dimeric GPVI fusion protein, is currently in phase 2 trials as an antithrombotic therapy.[4] Platelets are small circulating anucleate cell fragments that are essential for hemostasis, but platelets are increasingly recognized as mediators of a broad range of hematologic functions. Platelets have been shown to safeguard the integrity of developing and dysfunctional vessels under inflammatory conditions. GPVI was recently established as an essential mediator of vascular integrity in inflammatory settings.[5] The tumor microenvironment is one such setting. The study by Volz et al is the first to investigate this function of GPVI in solid tumors. Using both orthotopic and heterotopic models of tumor implantation in mice, they demonstrate increased intratumoral hemorrhage with either GPVI depletion in the host, or acute GPVI inhibition using an F(ab')$_2$ fragment of JAQ1, an antibody that blocks the major collagen binding site on murine GPVI. The results achieved are similar to those obtained with acute platelet depletion.[2,6] The treatments directed against GPVI also increased the accumulation of chemotherapeutic drugs in the tumors. With anticancer drugs given every 4 days, the authors observed additive effects of GPVI inhibition or platelet depletion on tumor growth suppression. This provides proof of concept for combined GPVI targeting with chemotherapeutic drugs as a potentially effective antitumor approach targeting specific platelet functions but with minimal bleeding complications.

Unlike most current antiplatelet antibodies, the JAQ1 F(ab')$_2$ fragment does not lead to platelet clearance. Thus, the ability of JAQ1 F(ab')$_2$ to induce intratumoral hemorrhage can be attributed to molecular blockade of GPVI on circulating platelets, although contributions of plasma GPVI shed from platelets cannot be ruled out. This in itself is a striking result, because it indicates that GPVI exposure is the principal mediator of platelet-dependent vascular integrity. Of particular note is that mechanisms of GPVI-dependent vascular integrity in inflammation appear to vary depending upon the extent of vascular damage and the underlying context.[7] In the case of small breaks in the endothelial barrier exposing subendothelial collagen and laminin, single platelets can plug the leak via GPVI engagement in many inflammatory settings. This may be the case in dysfunctional tumor vasculature.[8] Indeed, Volz et al were able to reproduce the hemorrhage and tumor growth inhibition of JAQ1 F(ab')$_2$ using soluble dimeric GPVI-Fc fusion protein, which competes for platelet-collagen binding, providing further support for this mechanism in the solid tumor models. However, GPVI inhibition caused massive intratumoral hemorrhage beyond what might be anticipated by single platelet-sized gaps in endothelium. Earlier studies demonstrated that infiltrating leukocytes are the major drivers of platelet-dependent intratumoral hemorrhage.[9] One possible explanation for the increased intratumoral hemorrhage in GPVI-blocked mice could involve multiple steps. GPVI is required initially to establish single platelet plugs via anchorage and spreading on subendothelial matrix. In the absence or blockade of GPVI, inflammatory cells, principally neutrophils, infiltrate and induce further vascular damage, thereby increasing the extent of hemorrhage, as observed by Volz et al. Neutrophil recruitment to the tumor microenvironment was not altered by GPVI inhibition, supporting a role for GPVI in either preventing or possibly repairing vascular damage induced from neutrophils. However, neutrophil depletion did not fully prevent hemorrhage by GPVI blockade, indicating contributions from other factors.