## Donor cell leukemia: is reappearance of gene mutations in donor cells more than an incidental phenomenon?

Acute myeloid leukemia (AML) is one of the extreme outcomes of age-related clonal hematopoiesis (ARCH).[1] With aging, mutations accumulate in hematopoietic stem and progenitor cells (HSPC).[2,3] Based on the estimated number of HSPC (~50,000) in the human body and the number of somatic mutations in single adult cells (~1000),[4] it is predicted that every ~100 nucleotides, a somatic mutation will occur at a low variant allele frequency (VAF). Indeed, recent studies have demonstrated that virtually all elderly healthy individuals carry protein-damaging mutations in *DNMT3A* and *TET2* at a low VAF.[5] Although mutations in preleukemic genes are inevitable, only 10-30% of older adults develop ARCH (large clone VAF >0.02).[6] Moreover, only one out of 1,000 carriers of somatic mutations will develop a myeloid malignancy. Recent studies have revealed that individuals with ARCH who carry larger clones and/or more than one mutation and specific mutations, are at a higher risk of developing AML. However, the underlying mechanisms resulting in clonal evolution that eventually leads to full-blown AML remain unclear.

HSPC reside in the bone marrow (BM), where they are supported by mesenchymal stromal cells that maintain their quiescence and/or promote their proliferation and differentiation. The BM microenvironment can determine whether HSPC carrying mutations will outgrow their wild-type counterparts. The role of the BM microenvironment in the induction of leukemia has been described in a rodent model,[7] but studies in humans are scarce.

We present here two rare cases of late-onset donor cell leukemia (DCL) that developed in allogeneic stem cell transplant (SCT) recipients and not in their sex-mismatched donors (Table 1). These findings might imply the involvement of the BM microenvironment in the initiation and evolution of myeloid malignancies.

The study was approved by the Institutional Review Board of Rambam Health Care Campus (approval n. 0016-15). The molecular profile of the patients was assessed using deep, error-corrected sequencing (average coverage ~5000X) as described in the *Online Supplementary Methods*. The presence of mutations (somatic and germline) at diagnosis and at the occurrence of DCL was evaluated (variants with VAF >0.1) in the patients' peripheral blood and saliva as well as in the donors' peripheral blood. Karyotype and chimerism analyses were performed in DCL samples. To confirm the presence of donor sex chromosomes and full donor chimerism, polymerase chain reaction (PCR) for short tandem repeats and fluorescence *in situ* hybridization (FISH) analyses were done (*Online Supplementary Table S1*). To explore the possibility that DCL originated from ARCH mutations present in the donor we applied ARCH variant calling algorithms aimed at accurately detecting recurrent AML mutations at a VAF >0.005. Additionally, we longitudinally followed complete blood count (CBC) parameters in 71 long-term survivors post-allogeneic SCT. These individuals were identified in the electronic database of a health maintenance organization encompassing 3.45 million people. The CBC of these patients were compared to those of 500,000 age- and gender-matched controls.

The current report describes the analysis of two patients with myelodysplastic syndrome (MDS)/AML who underwent allogeneic SCT from HLA-matched, sex-mismatched family donors and relapsed with DCL years after the procedure.

In the first case, a 54-year old male, diagnosed with AML in 2006, exhibited a mutation in the *U2AF1* S34F gene (*Online Supplementary Tables S1* and *S2*). The patient achieved complete remission and was transplanted from his HLA-matched sister (52 years old) who carried a mutation in the *DNMT3A* F752Y gene at a low VAF (2.96%) at the time of donation (*Online Supplementary Table S3*). Nine years later (2015) the patient relapsed, presenting with different mutations, i.e., *U2AF1* (S34Y instead of the original S34F), *IDH1* R132C and *DNMT3A* F752Y at higher VAF (43.3%). At the time of DCL onset he had full chimerism and 100% XX donor cells, as confirmed by PCR for short tandem repeates and FISH. Notably, the mutations in the *U2AF1* and IDH1 genes were not detected in the donor's peripheral blood either at the time of cell donation or 10 years later (Figure 1: case 1). To date, the donor remains in good general condition with normal CBC.

In the second case, a 26-year old female was diagnosed with high-risk MDS in 2000 and transplanted from her haplo-matched father (73 years old) (*Online Supplementary Tables S1* and *S2*). At the time the MDS was diagnosed a mutation in the *U2AF1* S34F gene was found. Seventeen years later (2017) the woman relapsed with high-risk MDS, presenting with mutations in *TET2* S1059X and *ASXL1* W1411X, not observed at diagnosis. At the onset of DCL the patient had full donor chimerism and 100% XY donor cells, as confirmed by PCR for short tandem repeats and FISH. These mutations were not detected in donor's peripheral blood during donation. (Figure 1, case 2; *Online Supplementary Table S3*). Notably, the donor died from a cerebrovascular accident.

In both analyzed DCL patients, the red blood cell distribution width (RDW) remained elevated even 9 and 17 years after the transplant (Figure 1). As increased RDW has been reported to be associated with dyserythropoiesis and a poor prognosis in patients with hematologic

**Table 1.** Potential confounders responsible for the clinical course after allogeneic stem cell transplantation.

| | Case 1 | Case 2 |
|---|---|---|
| Conditioning regimens | Reduced-intensity: fludarabine/melphalan | Myeloablative: total body irradiation/ fludarabine/thiotepa/ATG |
| GvHD prophylaxis | CSA + MTX | T-cell depletion |
| Acute GvHD grade | 2 | 3 |
| Chronic GvHD grade | Mild | Mild-moderate |
| Pharmacological immunosuppression after SCT | 1.5 years | 3 years |

ATG: antithymocyte globulin; CSA: cyclosporine; GvHD: graft-*versus*-host disease; MTX: methotrexate; SCT: stem cell transplantation; .

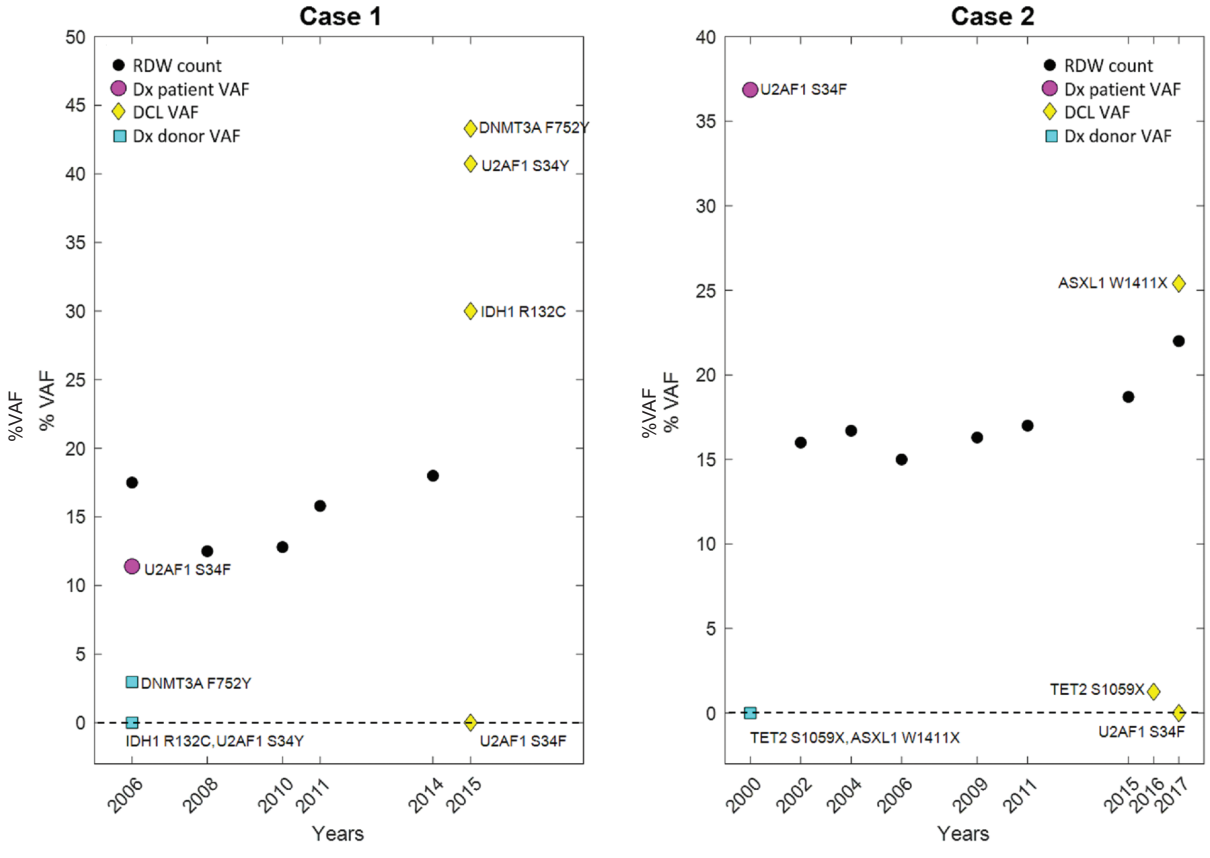


**Figure 1. The variant allele frequency profile and red cell distribution width of two patients with donor cell leukemia that developed long after transplantation for acute myeloid leukemia.** RDW: red cell distribution width; Dx: diagnosis; VAF: variant allele frequency; DCL: donor cell leukemia (DCL).

malignancies, the present study analyzed longitudinal CBC of another 71 allogeneic SCT recipients, recorded in the dataset of Clalit, a health maintenance organization (*Online Supplementary Methods*). While most CBC parameters, including RDW, gradually normalized in the majority of patients in this cohort over the first 4 years following allogeneic SCT, ~25% of these long-term survivors still exhibited high RDW. This evidence could be suggestive of underlying MDS, or other hematologic pathologies associated with increased RDW,[8] DCL being one of them.

Moreover, in an Italian study including 94 adults with acute leukemia, unfavorable cytogenetics were found in 26% of patients with high RDW compared to 8% of patients with RDW within the normal range ($P$=0.10).[8] Additionally, increased RDW was reported among individuals with ARCH[6] and specifically in those at a greater risk of AML development. This change in erythropoiesis might be mediated, at least in part, by an impaired BM microenvironment, although an abnormal crosstalk between cells in the BM and clonal HSPC (ARCH) could also be a possible contributor.[9] While a damaged microenvironment might be involved in both DCL evolution and RDW dynamics, the mechanisms underlying these effects may differ.

The long period between allogeneic SCT and DCL development observed in our patients could imply either that the evolution occurred in donor HSPC, originating from mutations that were scanty (below the detection limit) in the donor and then expanded in the recipient, or that new mutations were acquired and the mutated cells proliferated in the recipient only. In case 1, a preleukemic mutation (*DNMT3A*) was found in the donor at the time of donation.[10-12] While inherited predisposition to MDS/AML is known, it is less likely to be the case in the current patient, as no other leukemias have been reported among family members. Hence, the initiation of DCL in the cases reported here could have been triggered by involvement of the BM microenvironment in abnormal hematopoiesis.[13-15] This interpretation of ours is supported by the fact that in patient #1 the same gene has been affected twice in the same position, as revealed at diagnosis and in the transplanted donor's cells 9 years after SCT. Of note, the chance of the same patient developing AML twice in the same position within the gene (*U2AF1* S34) is less than 1:500,000.[2]

Overall, our findings suggest that at least in some MDS/AML cases, following allogeneic SCT, certain mutations in the donors' cells might have a selective advantage in specific conditions of the BM microenvironment. Hematopoiesis is not fully normalized as a result of abnormalities in the recipient's BM microenvironment, which trigger leukemogenesis in the donor's cells that may eventually lead to DCL. Better understanding of this interplay could shed light on the mechanisms of AML evolution and contribute to advances in prevention and treatment of this disease.

*Tal Shahar Gabay,[1,2] Noa Chapal-Ilani,[3] Yoni Moskovitz,[3] Tamir Biezuner,[3] Barak Oron,[3] Yardena Brilon,[3]*

*Anna Fridman-Dror,[1] Rawan Sabah,[1,2] Ran Balicer,[4] Amos Tanay,[3,5] Netta Mendelson-Cohen,[5] Eldad J. Dann,[6] Riva Fineman,[6] Nathali Kaushansky,[3] Shlomit Yehudai-Reshef,[1*#] Tsila Zuckerman[2,6#] and Liran I. Shlush[3,6#]*

*[1]Hematology Research Center, Rambam Health Care Campus, Haifa; [2]Bruce Rappaport Faculty of Medicine, Technion, Haifa; [3]Department of Immunology, Weizmann Institute of Science, Rehovot; [4]Clalit Research Institute, Tel Aviv; [5]Department of Computer Science and Applied Mathematics, Weizmann Institute of Science, Rehovot and [6]Department of Hematology and BMT, Rambam Health Care Campus, Haifa, Israel*

*[#]SY-R, TZ and LIS contributed equally as co-senior authors.*

*Correspondence:*
*TSILA ZUCKERMAN - t_zuckerman@rambam.health.gov.il*

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