Exome sequencing reveals heterogeneous clonal dynamics in donor cell myeloid neoplasms after stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an established treatment option for myeloid neoplasms (MN). In rare cases, the development of *de novo* hematologic malignancies derived from cells of donor origin, named donor cell hematologic neoplasm (DCHN), can occur.[1,2] Although some mechanisms have been suggested, the etiological factors and mechanisms involved in DCHN onset remain elusive. Moreover, DCHN is an extremely unusual allo-HSCT complication. But it provides a useful *in vivo* model to understand the genomic processes driving the leukemic transformation of donor stem cells. This study collects the first large cohort in which whole exome sequencing (WES) was performed in several post-transplant bone marrow (BM) samples from DCHN patients and their donors.

A cohort of seven patients (Table 1 and *Online Supplementary Appendix*) recruited from three hospitals belonging to the Spanish Group of Hematopoietic Transplantation (GETH) and their donors were included. The study included 32 BM samples from different time points after allo-HSCT (*Online Supplementary Figure S1*) as well as one peripheral blood (PB) sample from each donor. The research protocol was approved by the Ethics Committee of Gregorio Marañón General University Hospital. The study WES workflow is summarized in Figure 1 and the *Online Supplementary Methods*.

The results of the analysis of the mutational profiles obtained from the sequential post-HSCT samples demonstrated high intra-tumor genetic heterogeneity and clonal dynamics for all seven donor cell myeloid neoplasm (DCMN) cases (Figure 2). Among the altered genes, 27 variants in 26 strong candidates with oncogenic potential were found. This analysis showed 19 variants in genes associated with RNA processing and metabolism (*LUC7L2, NOP14*), cell differentiation (*LAMA5, SKOR2, EML1*), signal transduction (*SNX13, IRS1, TENM2*), including notch signaling pathway (*NOTCH4, DTX1*) and ERBB2 signaling pathway (*GRB7*), immunity regulator (*MEFV*), histone deacetylase (*GSE1*), DNA damage response (*PNKP*), post-translational modifications (*SENP7*), transcription factor (*TAF1L, ZKSCAN2, ZNF461*), and apoptotic process (*MEGF10*), as well as eight mutations in seven genes commonly found in adult acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) (*SETBP1, DNMT3A, TET2, RUNX1, CSF3R, EP300* and *IDH2*). All these mutations were confirmed by a customized targeted gene panel designed ad hoc to validate the variants selected in the WES which also included the most recurrent mutations in MN (*Online Supplementary Appendix*). By this approach, the gene panel analysis also allowed identification of mutations in *CSF3R, NPM1, TP53* and *ASXL1* genes (*Online Supplementary Table S1*).

Analysis of CNV revealed that the most common chromosomal alterations in DCMN were monosomy 7 (-7) or chromosome 7 abnormalities, which were detected in 6 out of 7 patients (Figure 2 and *Online Supplementary Figures S3-S9*).

Regarding the analysis performed on donor samples, copy number variation (CNV) analysis of stem cell apheresis from donor 1 revealed -7. Four years later, the recipient developed a donor cell myelodysplastic syndrome (DC-MDS), which showed -7 together with other molecular (*SETBP1, LUC7L2*) and cytogenetic alterations. This case highlights what has been described by other authors who suggested that *SETBP1* mutations occur at later stages of disease evolution, influencing the clinical course of the disease rather than its initiation; they are associated with a poor prognosis.

The other six donors presented variants in genes that have been involved in hematologic or solid tumors: *KLLN* (c.445T>A), *HOXD4* (c.242A>T), *MSR1* (c.877C>T), *HOXD12* (c.213T>C), *MOS* (c.426_432dupTGGCAAC), *SETBP1* (c.3962G>A), *MAD1L1* (c.851A>G).[3,4,5,6,7] Of note, variants present in

Table 1. Clinical features of donor cell myeloid neoplasm (DCMN) patients.

Patient	Age/Sex	Primary disease	Donor Age/Sex	Status pre-HSCT	Donor type	Stem cell source	Type of DCMN	Cytogenetics of DCMN	Time from allo-HSCT to DCMN (months)	Treatment	Outcome	Donor
1	56/M	MCL	72/M	2ªCR	MR	PB	MDS	45,XY,-7,del(12)(p12)	57	AZA + SCT	*Dead*	BM dysplasia
2	26/F	ALL	64/M	2ªCR	MMR*	BM	MDS	46,XY,del(5q),del(7q)	34	–	*Dead*	Healthy
3	39/M	CML-CP	49/M	NCR	MR	BM	MDS	45,XY,-7	249	AZA + SCT	Alive	Healthy
4	60/M	AML	55/F	1ªCR	MR	PB	AML	45,XX,t(3;16)(q21;q22),-7[16] 90-130,XXXX,t(3;16)(q21;q22), t(3;16)(q21;q22),-7,-7,+2-5 marc[2], 46,XX[2]	19	AZA + SCT	*Dead*	Healthy
5	55/M	MCL	59/M	1ªCR	MR	PB	AML	46,XY,del(7)(q31q36)// 47,XY,+1,der(1;7)(q10;p10)	67	AZA + SCT	*Dead*	Healthy
6	46/F	ALL	0/F	1ªCR	MU	CB	AML	46,XX	24	AZA + SCT	*Dead*	–
7	46/M	ALL	63/F	1ªCR	MMR*	BM	MDS	46,XX,t(10;11)(q24;p15)[13] 45,sl,-13,16,+mar[2] 46,sl,del(7)(q22)[5]	5	Hydroxyurea	*Dead*	Healthy

M: male; F: female; MCL: mantle cell lymphoma; ALL: lymphoblastic leukemia; AML: acute myeloid leukemia; CML-CP: chronic myelogenous leukemia *BCR-ABL1* positive-chronic phase; CR: complete remission; NCR: non-cytogenetic response; MDS: myelodysplastic syndrome; MR: matched related; MU: matched unrelated; MMR: mismatched related; PB: peripheral blood; CB: cord blood; CK: complex karyotype; AZA: azacitidine; HSCT: hematopoietic stem cell transplantation. *1-antigen HLA-mismatched. Patients were transplanted between 1994 to 2015. Donors were healthy at the time of DCMN diagnosis.

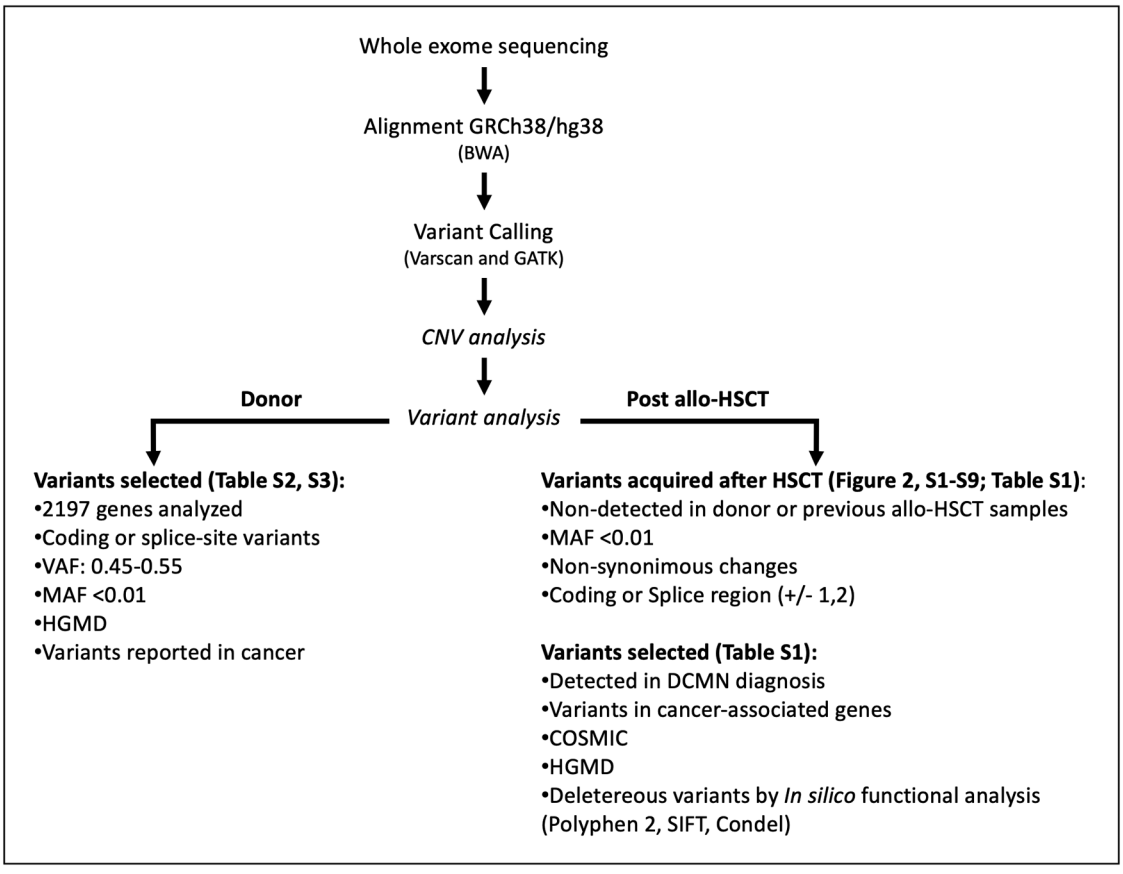

Figure 1. Flowchart of sequencing and filtering methods to identify and prioritize variants. Post-allogeneic hematopoietic stem cell transplantation (allo-HSCT) samples were matched against their donor peripheral blood (PB) and previous post allo-HSCT samples to identify the acquisition of mutations along post allo-HSCT period until donor cell myeloid neoplasm (DCMN) development. Those somatic variants detected in coding region and donor or acceptor splicing zone, missense, nonsense and frameshift variants with minor allele frequency (MAF) <0.01 were further studied. Copy number variation (CNV) analysis was performed in all samples. To perform the donor analysis a cancer-associated gene list was compiling, the list comprised of a total of 2197 genes and variants detected in coding or splice-site, with variant allele frequency (VAF) between 0.45-0.55, MAF <0.01 were further studied. BWA: Burrows-Wheeler Aligner; COSMIC: Catalogue Of Somatic Mutations In Cancer; HGMD: Human Gene Mutation Database). [1]See *Online Supplementary Tables S2* and *S3*. [2]See Figure 2, *Online Supplementary Table S1* and *Online Supplementary Figures S1-S9*.

donor stem cells were also detected in all the corresponding follow-up samples after allo-SCT. Based on the variant allele frequency (VAF), all variants seem to have germline origin except for those in *MOS* (donor 5) and that in *SETBP1* (donor 6), which appear be of somatic origin. Unfortunately, germline samples were not available to confirm this observation.

Interestingly, although acquired or inherited genetic alterations, which might be related to a predisposition to cancer, were observed in all donors, none of them had developed overt neoplasia at the moment of DCMN diagnosis in the recipients.

Considering the results in patients and donors described above, we propose a plausible model of leukemogenesis for each case, with progressive emergence of mutations in donor cells related to the development of AML or MDS after allo-HSCT (Figure 2). In cases 2, 4, 6 and 7, in which the median time to DCMN diagnosis was 21.5 months, the acquisition of mutations and the evolution of the leukemic clone occurs early after allo-HSCT, a period characterized by a marked immunosuppression state in the patients. Moreover, these four patients had received conditioning regimens based on chemotherapy combined with total body irradiation (TBI) and/or timoglobulin, which would also contribute to the development of MN.

The present study shows that MN evolve by an iterative process of genetic diversification derived from clonal selection and expansion that begin before the clinical onset, in accordance with the current multistep pathogenesis model of leukemogenesis.[8] After the initiating mutation, malignant clones evolve and accelerate disease progression through the acquisition of new mutations. Although no clear pattern of clonal mutation acquisition has been observed, initiating mutations appear to affect epigenetic regulators of transcription (*DNMT3A, TET2*) or genes involved in intracellular signal transduction (*SNX13, RHPN2, IRS1, CSF3R*).

Different factors can influence the development of a neoplasm in donor cells. Interestingly, cytogenetic abnormalities involving chromosome 7 were the most frequent in our DCMN cohort (6 of 7 patients). The frequency of -7 is particularly high among therapy or radiation-induced MDS or AML,[9] as well as *TP53* and epigenetic modifying gene somatic mutations,[10] as in 4 out of 7 patients. In the present cohort, 6 of 7 patients received an alkylating agent or antimetabolites within the conditioning regimen. Furthermore, four of them also received ionizing radiation therapy, and the time of leukemogenic transformation in these patients was shorter than in those who only received chemotherapy. Due to the high prevalence of chromosome 7 anomalies in this entity (a characteristic previously observed by others authors[2]), it seems that the residual

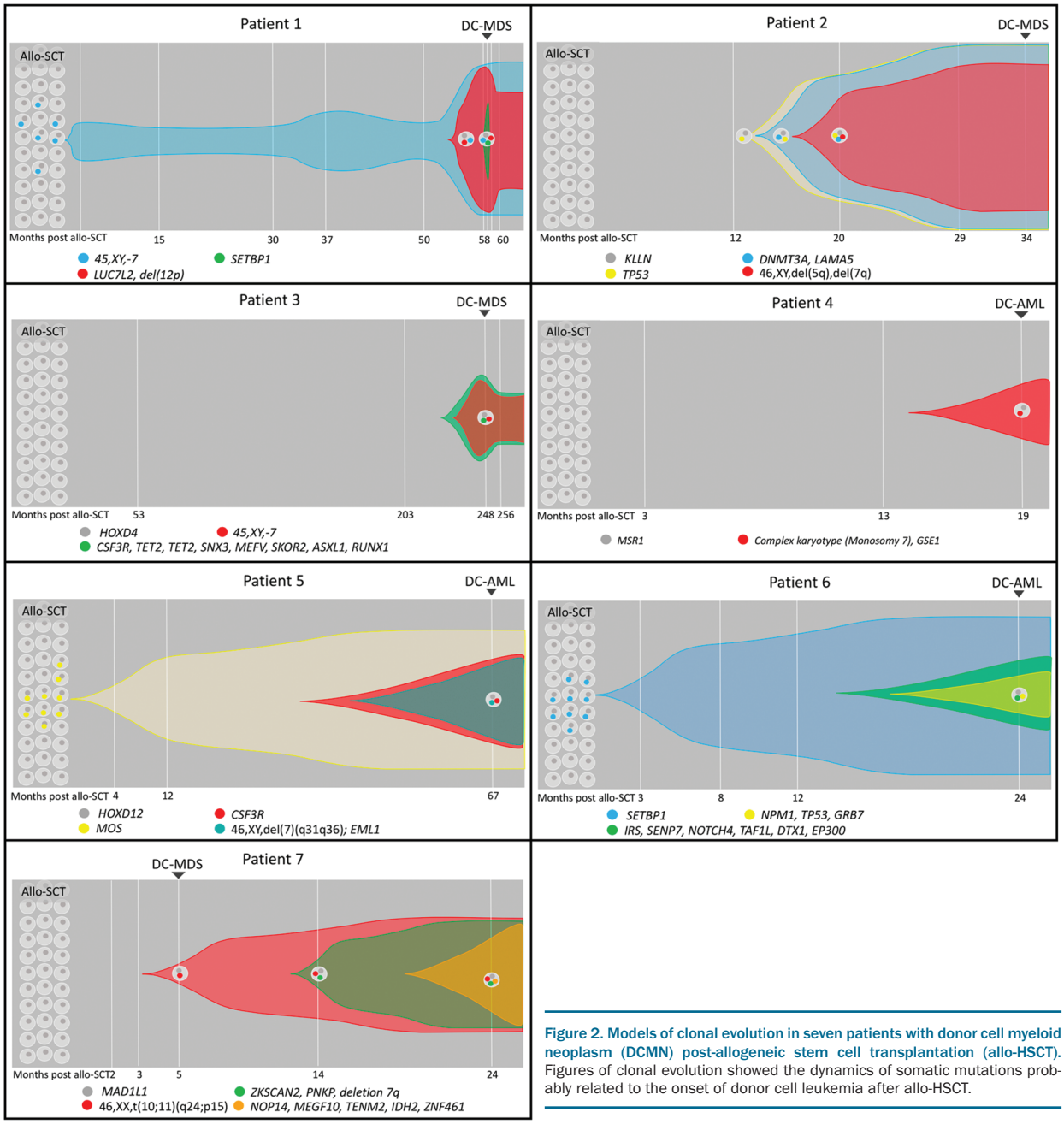

Figure 2. Models of clonal evolution in seven patients with donor cell myeloid neoplasm (DCMN) post-allogeneic stem cell transplantation (allo-HSCT). Figures of clonal evolution showed the dynamics of somatic mutations probably related to the onset of donor cell leukemia after allo-HSCT.

toxicity in the BM of prior chemotherapy and/or TBI plays an important role in pathophysiology of this disease. Of note, none of the donors had received chemotherapeutic or other toxic drugs.

Additionally, the post allo-HSCT period is characterized by a decreased immune surveillance caused by both conditioning regimens and by the immunosuppressive drugs administered to prevent graft-*versus*-host disease. Downregulation or inactivation of the immune system may facilitate malignant clonal progression. Consequently, the appearance or outgrowth of cells with potential to become cancerous is increased in this period.

Noteworthy, all seven donors in the present study had germline or acquired genetic alterations in genes which might be related to tumor development. Although some of the specific variants found in the donors have been described in low frequencies in particular human populations, all of them have also been previously described in families with a predisposition to cancer. Cancer-related genes usually show incomplete penetrance and variable expressivity, and need additional genomic events to lead to the development of a tumor.[11] In this regard, post-SCT conditions might contribute to the progression of the phenotype. The existence of an acquired premalignant state bearing the initiating lesions has been reported in some donors who have no other signs of disease. Moreover, somatic mutations in genes involved in leukemogenesis were found in 5%, 10% and 20% of 60-, 70-, and 90-year-old individuals, respectively.[12] Likewise, humans with clonal hematopoiesis have an increased risk of developing hematologic neoplasms compared with those without mutations.[13] However, the use of older donors with clonal hematopoiesis of indeterminate potential (CHIP), otherwise revealed as a safe approach, has recently been associ-

ated with an increased risk of developing DCMN.[14] Interestingly, CHIP-associated mutations were not found in any of the donors in our cohort, even though 5 of 7 were over 55 years old. This observation indicates that different mechanisms may be involved in the development of DCMN which would need further investigation.

Genetic predisposing factors are presumed to play an important role in the development of MN. Likewise, 16-21% of cancer survivors who developed t-MN have a germline mutation associated with inherited cancer susceptibility genes.[15] It could be that individuals with germline mutations in these genes are particularly susceptible to cytotoxic chemotherapy and/or radiation.

The growing use of next-generation sequencing (NGS) to study gene panels to define patient prognosis or identify targetable genomic alterations would allow detection of germline variants with clinical significance in tumor samples. Identification of germline mutations in the recipient could rule out the variant in family members. Relatives with the mutation would not be considered as donors and would eventually be referred for genetic counseling.

In most cases reported in the literature, the donor has no evidence of MN development.[2] Indeed, donors with such inherited molecular alterations do not necessarily develop leukemia, and they can live for many years without evidence of the disease, since other genetic or environmental factors, which are usually altered in transplanted patients, might play an important role in the development of malignant disease.

Presumably not just one mechanism from among those mentioned above is responsible for the leukemic transformation in donor cells. But a combination of various conditions would contribute to the development of DCMN after allo-HSCT. Such pre-leukemic stem cells from the donor might increase the likelihood that, later, co-operating mutations arise in cells that already contain the initiating mutations in a context of decreased immune surveillance. The relative contribution of each of the genetic and transplant-related factors to donor-derived leukemia is still not known. Novel approaches based on in-depth NGS to study consecutive samples from the post-transplant period in these patients appear promising to discover new genes involved in the development of MN and to decipher the ultimate mechanisms of leukemogenesis.

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