Clonal genetic evolution at relapse of favorable-risk acute myeloid leukemia with *NPM1* mutation is associated with phenotypic changes and worse outcomes

Acute myeloid leukemia (AML) is a dynamic disease caused by accumulating, somatically acquired driver mutations generating branching competing clones.[1] In favorable-risk AML, high resolution genomic profiling by single nucleotide polymorphism array analysis of paired diagnostic-relapse *NPM1*^{mut} and *CBF* AML samples revealed increased genomic complexity at relapse but most patients retained founding mutations.[2,3] Furthermore, it has been extensively reported that phenotypic changes are commonly found at relapse in AML patients. It seems plausible that clonal evolution could be reflected in the phenotypic shifts of AML blast cells found at relapse, although the correlation with genetic clonal evolution has not been established.[2,4-7] The aim of our work was to determine the patterns of genetic clonal evolution occurring from diagnosis to relapse in favorable-risk AML patients by tracking the kinetic behavior of the most frequent co-mutations in paired samples and correlating these with the occurrence of phenotype shifts on blast cells and with the clinical outcome.

We included a total of 26 patients with favorable-risk AML (non-promyelocytic), according to European LeukemiaNet criteria, who relapsed after achieving complete remission. These patients were treated with the intensive chemotherapy schedules standard at the time of diagnosis and experienced a relapse after a median of 17.5 months (range, 4-252) (Table 1). As controls, we studied seven *NPM1*^{mut} AML patients (median age: 46.7 years; range, 22-69) who achieved sustained complete remission after treatment with a median follow-up of 24 months and no evidence of leukemia relapse at last follow-up.

Bone marrow-derived genomic DNA was obtained from paired diagnostic-relapse samples. Details of the methods are available in the *Online Supplementary Material*. At diagnosis, among 16 *NPM1*^{mut} AML patients we found three cases with *DNMT3A*^{mut} (18.7%: two R882H and one new mutation, c.2705_2706delTC), two cases with *IDH1*^{mut} (12.5%), two cases with *IDH2*^{mut} (12.5%) and one case with *FLT3*-TKD^{mut} (c.2503>T, at low ratio: 0.18). No mutations in *RAS* and *TP53* were

Table 1. Clinical and biological data of 26 patients with favorable-risk acute myeloid leukemia.

Pt.	Center	Diagnosis	Age	Sex	WBC	Blasts*	FAB	Karyotype	Molecular	Transplant at 1st CR	Relapse	RFS (days)	Alive	OS after relapse	Treatment at relapse
1	HURS	1.02.08	47	M	55.5	90	M4	46XY	NPM1^{MUT}	AUTO	16.10.09	623	Yes	87	ALLO
2	HURS	14.05.11	39	F	145.0	92	M5a	46XX	NPM1^{MUT}	ALLO	16.11.11	186	No	5	AZA
3	HURS	1.05.90	54	F	NA	NA	M2	46XX	NPM1^{MUT}	No	10.05.11	7679	No	38	CT
4	HURS	31.10.11	61	F	7.1	58	M2	46XX	NPM1^{MUT}	AUTO	10.06.13	696	Yes	43	ALLO
5	HURS	31.05.09	47	M	4.9	85	M1	46XY, t(8;21)	RUNX1-RUNXT1	AUTO	16.12.10	564	No	26	ALLO
6	HURS	2.11.11	71	M	2.9	53	M2	46XY,t (8;21)	RUNX1-RUNXT1	No	21.05.12	201	No	3	No
7	HURS	6.03.07	52	F	72.6	80	M2	46XX	NPM1^{MUT}	AUTO	4.01.08	304	No	7	CT
8	HURS	16.06.07	65	M	69.9	98	M4	46XY	NPM1^{MUT}	ALLO	21.07.08	401	No	15	AZA
9	HURS	23.02.09	32	M	15.0	39	M1	46XY	NPM1^{MUT}	ALLO	9.09.09	198	No	5	AZA
10	HURS	4.05.12	64	F	61.3	92	M5a	46XX	NPM1 ^{MUT}	ALLO	10.12.12	220	No	1	No
11	HPTV	19.08.11	39	M	NA	54	M1	46XY	NPM1^{MUT}	No	29.12.11	132	No	1	No
12	HPTV	10.04.12	28	M	8.8	80	M2	46XY,inv16	CBFb/MYH1	AUTO	18.06.13	434	Yes	43	ALLO
13	HPTV	21.1.13	57	M	149.0	35	M1	46XY	NPM1^{MUT}	No	2.01.14	365	Yes	36	ALLO
14	HMM	25.02.09	13	F	2.1	75	M1	46XX	CEBPA^{MUT}	ALLO	26.07.12	1247	Yes	54	2nd ALLO
15	HMM	13.05.09	46	M	19.1	32	M4	46XY	NPM1^{MUT}	AUTO	12.01.12	974	Yes	60	ALLO
16	HMM	25.02.12	66	F	16.6	52	M4	46XX	NPM1^{MUT}	NO	12.11.13	536	Yes	38	ALLO
17	HMM	10.07.02	65	F	17.0	35	M2	46XX,t (8;21)	RUNX1-RUNXT1	No	09.01.13	183	No	5	CT-AZA
18	HMM	14.05.13	21	M	1.4	58	M2	46XY,t (8;21)	RUNX1-RUNXT1	ALLO	26.12.13	226	No	2	AZA
19	HMM	08.11.13	46	F	14.4	59	M4	46XX	NPM1^{MUT}	ALLO	28.04.15	536	No	1	CT
20	HMM	07.02.14	14	F	77.0	80	M2	46,XX, inv16	CBFb/MYH1	No	30.09.15	600	Yes	16	ALLO
21	HLF	16.06.04	52	M	4	83	M0	46XY	FLT3/NPM1^{MUT}	ALLO	28.01.10	2052	No	0	CT-AZA
22	HLF	12.08.08	32	F	1.7	100	M1	46XX	NPM1^{MUT}	ALLO	10.02.11	912	Yes	71	2nd-3rd ALLO
23	HLF	14.07.08	37	M	110	55	M4	46XY,inv16	CBFb/MYH1	No	5.03.09	234	Yes	94	ALLO
24	HLF	27.06.09	64	F	14.3	NA	M4	46,XX, inv16	CBFb/MYH1	AUTO	17.06.11	720	Yes	67	CT
25	HLF	11.04.11	60	M	110.9	NA	M2	46XY	CEBPA	ALLO	27.05.13	777	No	16	RIC-ALLO
26	HLF	4.04.07	68	F	21.4	63	M4	46XX	NPM1^{MUT}	No	12.08.08	496	No	4	CT

Pt: patient, HURS: University Hospital Reina Sofía (Córdoba); HPTV: Policlinico di Tor Vergata (Rome); HMM: University Hospital Morales Meseguer (Murcia); HLF: University Hospital La Fe (Valencia); M: male; F: female; WBC white blood cell count (x10^9/L); NA: not available; FAB: French-American-British; *: blasts in bone marrow; Mut: mutated; CR: complete remission; AUTO: autologous; ALLO: allogeneic; RFS: relapse-free survival; OS: overall survival; RIC: reduced intensity conditioning; CT: chemotherapy; AZA: azacitidine.

found. In contrast, seven non-relapsing *NPM1*^{mut} controls showed less genetic complexity: we detected only one case with *IDH1*^{mut} (14.3%) and no *DNMT3A*^{mut} was detected. With regards to *CBF*-AML (n=8), we detected one case with *DNMT3A*^{mut} (12.5%) and one with *C-KIT*^{mut} (12.5%) and no mutations were found in two *CEBPA* patients. At the time of relapse, two patterns of genetic findings were observed: 'no clonal evolution', with persistence of mutations of the original founding clone, and 'clonal evolution', with changes in the gene mutation profile. No clonal evolution was found in 20 patients (77%): ten from the *NPM1*^{mut} AML group (62.5%), all eight of the *CBF*-AML group (100%) and both of those with *CEBPA*-AML (100%). In ten *NPM1*^{mut} AML patients, *IDH*^{mut} and *DNMT3A* remained stable with the same variant allelic fraction (VAF) and no acquisition of *TP53*^{mut} was detected. In the *CBF*-AML and *CEBPA* groups, *DNMT3A*^{mut} and *CKIT*^{mut} remained stable at relapse and acquisition of *TP53*^{mut} was not observed (Figure 1A). Interestingly, the second pattern, clonal evoluation, was found in the remaining six patients (23%), all of who were in the *NPM1*^{mut} AML group (36.5%): loss of *NPM1*^{mut} was confirmed in four cases, evolution of *DNMT3A*^{mut} in two cases [one R882H (VAF of 7.1% to 49.1%) and one new mutation p.D876Y (VAF of 0 to 48.4%)], one patient acquired *FLT3*-ITD and one patient lost a previously present *FLT3*-TKD^{mut}. Absence of these newly acquired mutations (2 *DNMT3A* and 1 *FLT3*-ITD) in diagnostic samples

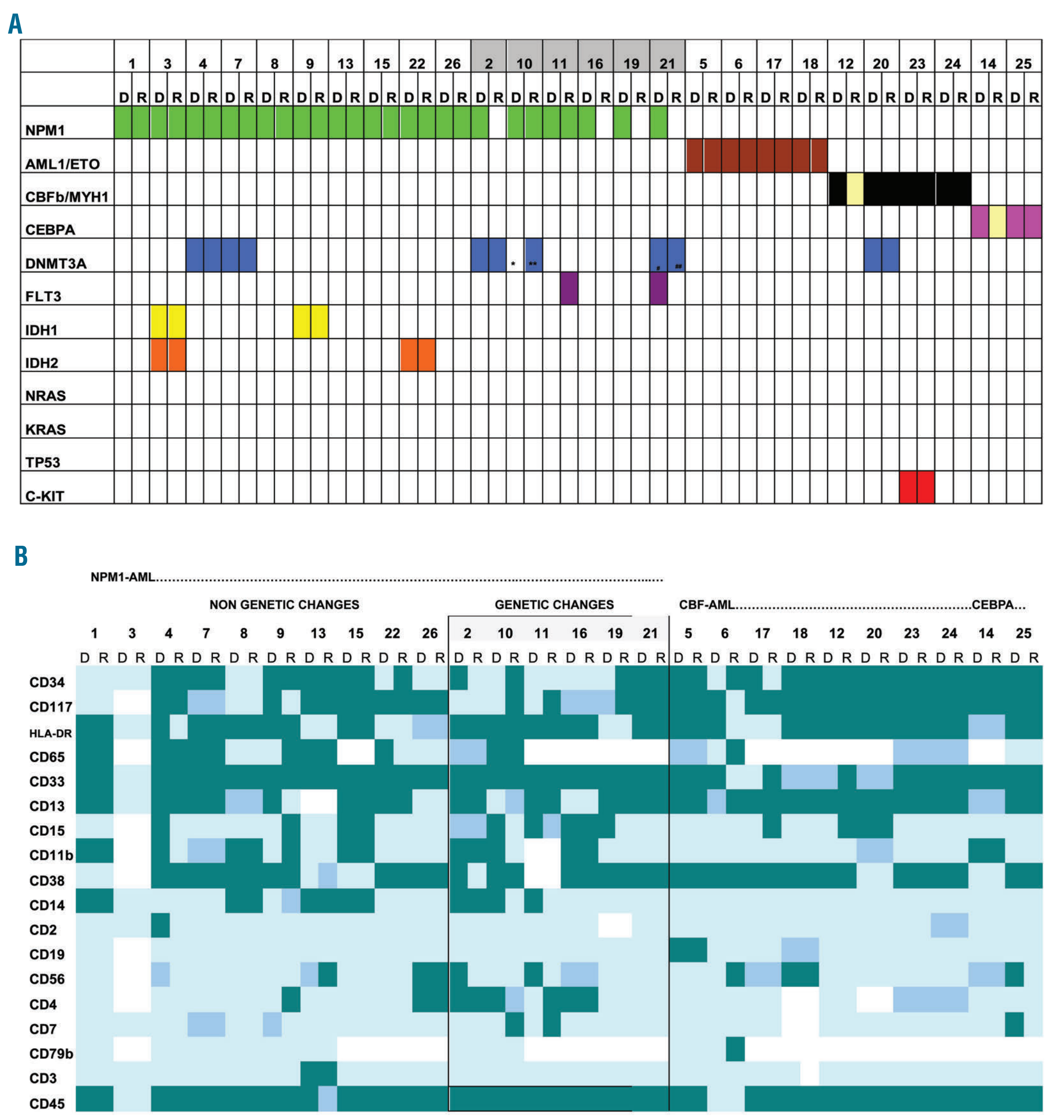

Figure 1. Clonal behavior in a series of 26 relapsing patients with favorable-risk acute myeloid leukemia. (A) Mutation analyses in paired diagnostic (D) and relapse (R) samples in 26 patients. Each column represents an individual patient. Colored bars indicate the presence of a mutation, blank bars represent wild-type for the specific gene and beige bars indicate that data are not available. *VAF 0%; **VAF: 48.4%; #VAF 7.1%; ## VAF 44.8%. (B) Immunophenotypic patterns in paired diagnostic (D)/ relapse (R) samples from 26 patients. Each column represents an individual patient. Colored bars indicate strong, dim and negative CD antigen detection for each marker. "Strong" means greater than 10^4, "dim" means between 10^3 and 10^4 and "negative" means lower than 10^3. Blank bars indicate missing data.

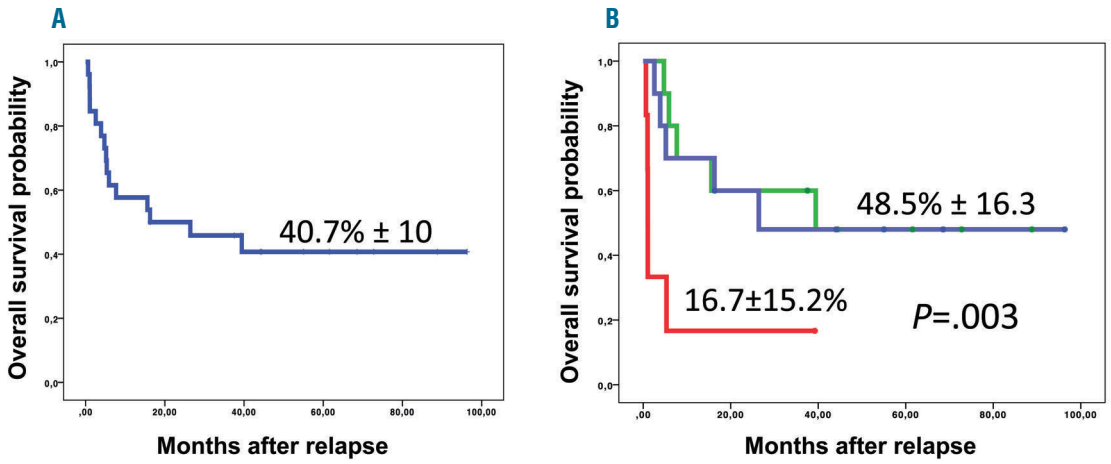

Figure 2.- Overall survival. (A) Overall survival of the whole series (n=26). (B) Overall survival comparing patients with *NPM1* and clonal evolution (red), *NPM1* without clonal evolution (green) and *CBF/CEBPA* without clonal evolution (blue).

was confirmed by next-generation sequencing as well as by reverse transcriptase polymerase chain reaction analysis (Figure 1A). By quantitative pyrosequencing analysis we demonstrated that both new *DNMT3A* mutations (c.2705_2706delTC,p.F902fs from patient 7 and c.2626G>T,p.D876Y from patient 10) were only found in leukemic samples and were not present in bone marrow samples obtained from patients in complete remission or in healthy donors *(Online Supplementary Figure S1*). From *in silico* studies, both mutations could alter normal function of native DNMT3A decreasing the activity of DNA methylation (*Online Supplementary Figure S2*).

For immunophenotypic analyses, at least 30,000 leukemic events were acquired, mostly in FACSCalibur or FACSCanto II dual-triple laser flow cytometers, and list modes files were analyzed with CellQuest™, FACSDiva™ or Paint-a-Gate software (Becton Dickinson Biosciences). Multidimensional analyses of immunophenotypes obtained at diagnosis and relapse were performed using the File merge and Automatic Population Separator functions of Infinicyt software (Cytognos SL, Salamanca, Spain). At diagnosis, most *NPM1*^{mut} AML patients displayed strong CD33 and CD13 expression (93.8% and 62.5%, respectively) with strong CD117 and CD34 expression in 43.8% and aberrant CD56 in 12.5%. *CBF*-AML blast cells expressed CD117 strongly in all cases, CD34 in 87.5%, CD56 and aberrant CD19 in 12.5% of cases. At the time of relapse, complete stability in the expression of all markers was observed in 14 patients (53.8%). By contrast, phenotypic profile evolution (defined as a significant modification of intensity in at least one marker) was confirmed in 12 patients (46%): eight of the 16 *NPM1*^{mut} patients (50%) and four of the group of ten with no *NPM1*^{mut} (40%). More frequently shifted expression was observed in CD15 (58.3% of patients), CD117, CD34 and CD56 (41.6%), CD7 and CD13 (33.3%), CD11b, CD4, CD33 and CD14 (25%) (Figure 1B). When comparing the incidence of phenotypic shifts in both genetic groups, we found that a significant percentage of patients with the 'no clonal evolution' pattern still displayed phenotypic shifts (8 out of 20; 40%) and this percentage was even higher among those showing a pattern of clonal evolution (4 out of 6; 66.7%), although the difference was not statistically significant (P=0.3). Altogether, 12 (46.1%) favorable-risk AML patients relapsed maintaining the same mutational and phenotypic profiles. A representative case of phenotypic shift is shown in *Online Supplementary Figure S3*.

Finally, we analyzed the impact of genetic patterns and phenotypic shifts on outcomes. At the time of analysis 11 patients were alive and in complete remission. The median follow-up after leukemia relapse was 55 months (range, 16.3-96.3) and probability of overall survival was 40.7% ± 10 for the overall series (Figure 2A). Salvage rescue treatment included allogeneic stem cell transplantation after re-induction chemotherapy (n=12; 46.1%) and intensive chemotherapy ± azacitidine (n=11; 42.3%) whereas three patients received only supportive care. Patients who underwent allogeneic stem cell transplantation had a statistical significantly higher probability of overall survival (82.5 ± 11.3% *versus* 7.1 ± 6.9, P<0.01). The median time from complete remission to relapse was shorter in patients with clonal evolution [12.6 (range, 6-67) months *versus* 18.5 (range, 6-252) months] than in the 'no-clonal evolution' group. Considering only the *NPM1*^{mut} group (n=16), 66.7% of patients showing clonal evolution had undergone allogeneic stem cell transplantation at first complete remission (4 out of 6; 66.7%) compared to 20% (2 out of 10) in the 'no-clonal evolution' group (*Online Supplementary Table S1*). Importantly, favorable-risk AML patients with no clonal evolution at relapse had a significantly higher estimated probability of overall survival compared to that of the group with clonal evolution (48.5 ± 11.5% *versus* 16.7 ± 15.2%, P=0.003) with a longer, mean estimated overall survival of 53.6 months (95% CI: 34.8;72.4) *versus* 8 months (95% CI: 0;19.3), respectively. Of note, overall survival probability was identical (48.5% ± 16.3) for AML patients with or without *NPM1*^{mut} within the 'no-clonal evolution' group (Figure 2B). In the multivariate analysis, only clonal evolution remained a significant adverse factor and allogeneic stem cell transplantation as salvage treatment of relapse as a favorable clinical factor (*Online Supplementary Table S2*).

In this study, we addressed genotypic and phenotypic clonal behavior in a series of 26 relapsing favorable-risk

AML patients. Our study demonstrated that the main scenario for leukemia relapse is the re-emergence of a founder clone with no clonal evolution (77% of cases), although 40% of such cases displayed phenotypic changes. This finding suggests that conventional chemotherapy protocols may not be able to achieve complete eradication of the founder AML clone, which is capable of regenerating the bulk of leukemic blasts after a variable period of time. This is in agreement with previous reports of genomic profiling studies by single nucleotide polymorphism arrays in AML series including all-risk subtypes or *NPM1*^{mut} cases[2,8] demonstrating increasing genomic complexity at relapse, which showed significantly worse outcomes[2,3] but maintenance of a common ancestral founder clone. Our data suggest the persistence of a rare subset of leukemic stem cells in favorable-risk AML after achievement of complete remission. These leukemic stem cells are capable of remaining quiescent for long periods,[9] such as in patient 3 who relapsed with the same genetic and phenotypic profile 20 years after achieving complete remission. In our series, all *CBF*-AML showed mutational stability, despite displaying phenotypic changes in 40% of cases. By contrast, clonal evolution was present in 36.5% of *NPM1*^{mut} AML and 66% of these cases also displayed phenotypic shifts. In our series, loss of *NPM1*^{mut} at relapse was the most frequent genetic evolution, followed by the acquisition of *DNMT3*^{mut}. Loss of *NPM1* at relapse was confirmed in four cases (25% of 16 *NPM1* cases). Three of them had undergone allogeneic stem cell transplantation at first complete remission and received azacitidine and/or chemotherapy with dismal outcome. These cases can plausibly be considered as "secondary therapy-related" or "clonally unrelated" AML. Importantly, our findings suggest that monitoring for minimal residual disease can be hampered by frequent phenotypic changes and also by the possibility of *NPM1*^{mut} losses. Minimal residual disease monitoring by multiflow cytometry or quantitative reverse transcriptase polymerase chain reaction for a genetic marker[11] can, therefore, be complementary and parallel monitoring could be quite useful to avoid false-negative minimal residual disease results, providing useful biological information to trace clonal evolution.[12,13]

Strikingly, in our series, *DNMT3A*^{mut} evolved in two patients, one of whom had concurrent loss of *NPM1*^{mut}. These findings, also in accordance with those reported by Krönke *et al.*,[2] point out the kinetic complexity of the interactions of *DNMT3A*^{mut} and *NPM1*^{mut} in AML patients at relapse, in whom new mutations in this epigenetic modifier occur as a "late event" in some instance.[2,10]

In conclusion, a comprehensive assessment of genetic and phenotypic features at relapse in favorable-risk AML provides useful biological information and could have important prognostic implications.

Carmen Martínez-Losada,[1] Juana Serrano-López,[1] Josefina Serrano-López,[1] Nelida I. Noguera,[2,3] Eduardo Garza,[4] Liliana Piredda,[2] Serena Lavorgna,[2] María Antonietta Irno Consalvo,[2] Tiziana Ottone,[2] Valentina Alfonso,[2] Juan Ramón Peinado,[5] María Victoria Garcia-Ortiz,[6] Teresa Morales-Ruiz,[6] Andrés Jérez,[7] Ana María Hurtado,[7] Pau Montesinos,[8] José Cervera,[8] Esperanza Such,[8] Marian Ibañez,[8] Amparo Sempere,[8] Miguel Ángel Sanz,[8] Francesco Lo-Coco[2,3] and Joaquín Sánchez-García[1]

[1]*Hematology Department, Reina Sofia University Hospital/Maimonides Biomedical Research Institute of Córdoba (IMIBIC)/University of Córdoba, Spain;* [2]*Department of Biomedicine and Prevention, "Tor Vergata" University, Rome, Italy;* [3]*Laboratory of Neuro-Oncohematology, Santa Lucia Foundation, Rome, Italy;* [4]*Laboratorios Dr. Moreira, Monterrey, Mexico;* [5]*Medical Sciences Department, Faculty of Medicine/University of Ciudad Real (UCLM), Spain;* [6]*Maimónides Biomedical Research Institute of Córdoba (IMIBIC)/University of Córdoba/ Reina Sofia University Hospital, Spain;* [7]*Hematology Department University Hospital Morales Meseguer-IMIB and* [8]*Hematology Department, Hospital Universitari I Politècnic La Fe, Valencia, Spain*

Acknowledgments: we thank Dr. Antonio Martinez-Peinado from the Clinical Analysis Department, University Hospital Reina Sofia (Córdoba, Spain) for technical support with Sanger sequencing. We are also grateful to Oskar Martínez from Health in Code Fundation (La Coruña, Spain) for technical support with next-generation sequencing and Maria Saarela for editing the manuscript.

Funding: this work was supported in part by a fellowship grant from Fundación Alfonso Martin Escudero (Madrid, Spain).

Correspondence: juanitatolea@yahoo.es

doi:10.3324/haematol.2018.188433

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-510.
2. Krönke J, Bullinger L, Teleanu V, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. Blood. 2013;122(1):100-108.
3. Sood R, Hansen NF, Donovan FX, et al. Somatic mutational landscape of AML with inv(16) or t(8;21) identifies patterns of clonal evolution in relapse leukemia. Leukemia. 2016;30(2):501-504.
4. Ho TC, LaMere M, Stevens BM, et al. Evolution of acute myelogenous leukemia stem cell properties after treatment and progression. Blood. 2016;128(13):1671-1678.
5. Ferrell PB Jr, Diggins KE, Polikowsky HG, Mohan SR, Seegmiller AC, Irish JM. High-dimensional analysis of acute myeloid leukemia reveals phenotypic changes in persistent cells during induction therapy. Plos One. 2016;11(4):e0153207.
6. Cui W, Zhang D, Cunningham MT, Tilzer L. Leukemia-associated aberrant immunophenotype in patients with acute myeloid leukemia: changes at refractory disease or first relapse and clinicopathological findings. Int J Lab Hematol. 2014;36(6):636-649.
7. Yebenes-Ramirez M, Serrano J, Martinez-Losada MC, Sanchez-Garcia J. Clinical and biological pronostic factors in relapsed acute myeloid leukemia patients. Med Clin (Bar). 2016;147(5):185-191.
8. Parkin B, Ouillette P, Li Y, et al. Clonal evolution and devolution after chemotherapy in adult acute myelogenous leukemia. Blood. 2013;121(2):369-377.
9. Thomas D, Majeti R. Biology and relevance of human acute myeloid leukemia stem cells. Blood. 2017;129(12):1577-1585.
10. Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI, Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. Leukemia. 2017;31(4):798-807.
11. Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424-447.
12. Hourigan CS, Gale RP, Gormley NJ, Ossenkoppele GJ, Walter RB. Measurable residual disease testing in acute myeloid leukaemia. Leukemia. 2017;31(7):1482-1490.
13. Getta BM, Devlin SM, Levine RL, et al. Multicolor flow cytometry and multigene next-generation sequencing are complementary and highly predictive for relapse in acute myeloid leukemia after allogeneic transplantation. Biol Blood Marrow Transplant. 2017;23(7):1064-1071.