BNIP3L in myelodysplastic syndromes and acute myeloid leukemia: impact on disease outcome and cellular response to decitabine

Changes in apoptosis of hematopoietic progenitors are thought to contribute to the progression of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).[1] BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L) are closely related members of the BCL2 family of proteins capable of inducing cell responses that may lead to apoptosis and/or autophagy.[2] Due to this dual role in cell death and survival, BNIP3 and BNIP3L have been described to execute both oncogenic and tumor suppressor activities.[2] The downregulation of *BNIP3* and *BNIP3L* are related to their aberrant methylation in acute leukemia and lymphoma,[3,4] respectively. However, they have been poorly investigated in myeloid malignancies. Herein, we evaluated the expression and methylation status of *BNIP3* and *BNIP3L* in MDS and AML, their impact on MDS prognosis, and their participation in the induction of apoptosis by decitabine.

BNIP3 and *BNIP3L* expression was evaluated by quantitative PCR in bone marrow samples from patients with MDS (n=65), AML with myelodysplasia-related changes (AML-MRC) (n=13), *de novo* AML (n=61) and from healthy donors (n=26). Comparisons were performed with a Mann-Whitney test. MDS patients were stratified according to the WHO 2008 classification into two groups: RA/RARS/del(5q)/RCMD and RAEB-1/RAEB-2. This study was approved by the local Ethics Committee. All patients were untreated at the time of sample collection and their characteristics are described in the *Online Supplementary Table S1*.

BNIP3 transcripts were significantly reduced in the RA/RARS/del(5q)/RCMD MDS group and in the RAEB-1/RAEB-2 MDS group in comparison with healthy donors, P=0.013 and P=0.001, respectively. AML-MRC and *de novo* AML patients presented no changes in *BNIP3* expression (Figure 1A). *BNIP3L* was decreased in the RAEB-1/RAEB-2 MDS group in comparison to healthy donors, P=0.018. A significant reduction of *BNIP3L* transcripts was also

observed in AML-MRC and in *de novo* AML compared with healthy donors, P=0.005, P=0.0003, respectively (Figure 1B).

BNIP3 and BNIP3L are regulated by *HIF-1α*,[5] which has been proposed to function as a tumor suppressor in AML.[6,7] Therefore, *HIF-1α* transcript levels were also evaluated in our cohort of patients. *HIF-1α* expression was reduced in the RA/RARS/del(5q)/RCMD and in the RAEB-1/RAEB-2 MDS groups compared to healthy donors, P=0.007 and P=0.014, respectively. Patients with *de novo* AML also presented decreased levels of *HIF-1α* in comparison with healthy donors (P=0.0007) (Figure 1C). Spearman´s rank correlation coefficient indicated a moderate correlation between *HIF-1α* and *BNIP3L* (r=0.41, P<0.0001), but not with *BNIP3*, in patient bone marrow samples (Figure 1D). Bone marrow samples from five MDS patients from our cohort who progressed according to the WHO 2008 classification were analyzed before and after progression. Interestingly, *BNIP3L* expression decreased after the progression of four of these patients (Figure 1E). *BNIP3* and *HIF-1α* expression varied after progression of these patients (*data not shown*).

A Cox regression model was used to estimate overall survival (OS) and event-free survival (EFS) of MDS patients. OS and EFS were defined as previously described.[8,9] With an average follow-up of 37 months, univariate analysis showed that lower levels of *BNIP3L* (below the median) negatively impacted OS, along with IPSS and advanced age (all P<0.05). Multivariate analysis showed that lower *BNIP3L* expression was an independent prognostic factor for worse OS (all P<0.05). As expected, IPSS and advanced age remained as independent predictors for OS. Lower levels of *BNIP3L* and IPSS also appeared as independent prognostic factors for worse EFS (all P<0.05). Kaplan-Meier analysis indicated a 5-year OS of 66% *versus* 32% for MDS patients with a higher *BNIP3L* expression *versus* a lower *BNIP3L* expression, respectively (Figure 1F). Similar results were observed for 5-year EFS (*data not shown*). Neither BNIP3 nor *HIF-1α* expression affected the survival outcomes of MDS patients (Table 1).

Investigation of the methylation status of *BNIP3* and *BNIP3L* promoter regions was performed as previously

Table 1. Univariate and Multivariate analyses of survival outcomes for MDS patients.

| | Univariate analysis | | | | | | Multivariate analysis | | | | | |
| | Event-Free Survival | | | Overall Survival | | | Event-Free Survival | | | Overall Survival | | |
Factor	HR[1]	(95% C.I.)	*P*	HR	(95% C.I.)	*P*	HR	(95% C.I.)	*P*	HR	(95% C.I.)	*P*
Sex Male *vs.* female	1.64	0.76-3.52	0.20	1.69	0.76-3.74	0.19	–	–	–	–	–	–
Age at sampling ≥ 60 *vs.* <60	2.90	1.002-8.39	0.04	3.66	1.09-12.24	0.03	–	–	–	4.50	1.14-17.71	0.03
IPSS risk group Int-2/High *vs.* Int-1/Low	10.79	4.26-27.31	<0.0001	8.74	3.33-22.93	<0.0001	11.80	3.57-38.98	<0.0001	12.70	3.24-49.69	0.0003
BNIP3 expression Low *vs.* high expression[2]	1.39	0.67-2.86	0.36	1.36	0.64-2.87	0.41	–	–	–	–	–	–
Numerical values	0.93	0.82-1.04	0.23	0.93	0.83-1.05	0.29	–	–	–	–	–	–
BNIP3L expression Low *vs*. high expression[2]	1.86	0.85-4.07	0.12	2.32	1.01-5.35	0.04	3.05	1.23-7.58	0.01	5.82	1.95-17.40	0.001
Numerical values	0.82	0.63-1.06	0.13	0.76	0.56-1.02	0.07	–	–	–	–	–	–
HIF1a expression Low *vs.* high expression[2]	0.82	0.36-1.83	0.62	0.77	0.33-1.79	0.55	–	–	–	–	–	–
Numerical values	0.33	0.03-3.58	0.36	0.37	0.03-4.27	0.43	–	–	–	-	–	–

MDS: myelodysplastic syndromes; C.I.: confidence interval; HR: hazard ratio; Int-1: intermediate-1; Int-2: intermediate-2; IPSS, International Prognostic Scoring System. [1]Hazard ratios >1 indicate that the first factor has the poorer outcome. 2 MDS patients were categorized as low gene expression (below median) and high gene expression (above median).

described.[3,4] *BNIP3* methylation was observed in 9% (3/33) RA/RARS/del(5q)/RCMD MDS, 17% (2/12) RAEB-1/RAEB-2 MDS, 17% (1/6) AML-MRC and 18% (7/39) *de novo* AML patients, and 0/13 healthy donors. *BNIP3L* methylation was investigated in a total of 7 heathy donors and 37 patients (RA/RARS/del(5q)/RCMD MDS =12, RAEB-1/RAEB-2 MDS =3, AML-MRC =2 and *de novo* AML =20), and was detected in 6 *de novo* AML patients, 1 RCMD and 1 RAEB MDS patient (Figure 1G). Part of these

samples were randomly analyzed for *BNIP3* (n=20) and *BNIP3L* (n=17) methylation by high resolution melting, and the same results were observed (*data not shown*).

Despite the low frequency of *BNIP3* and *BNIP3L* methylation in MDS and AML, we evaluated their gene expression in leukemia cells treated with the hypomethylating agent decitabine. Decitabine is one of the standard treatments for high-risk MDS[1] and has been considered for some cases of AML,[10] but the hematological response is
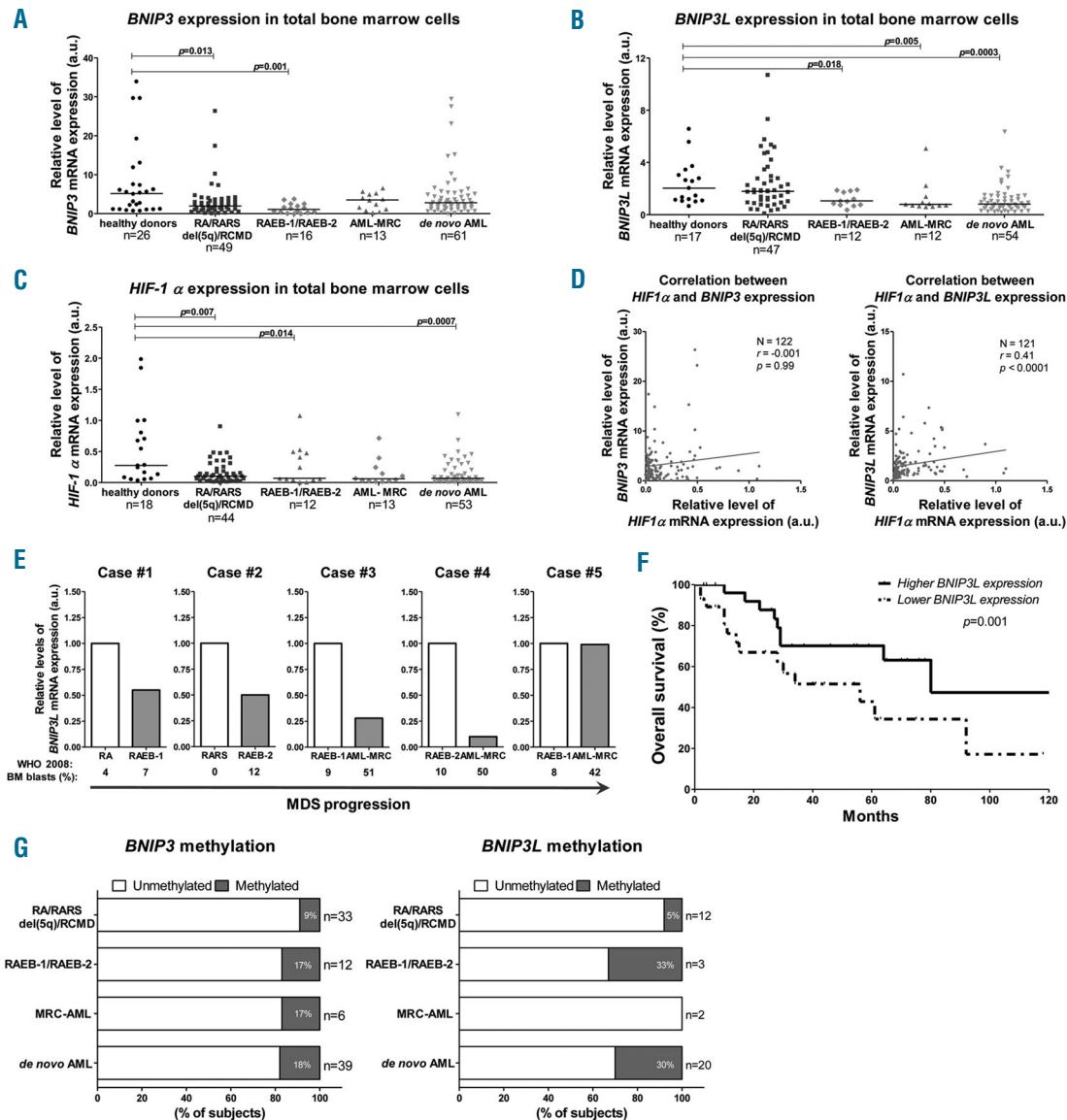

Figure 1. *BNIP3* and *BNIP3L* expression and methylation status in bone marrow cells from MDS and AML patients. *BNIP3* (A), *BNIP3L* (B) and *HIF-1α* (C) mRNA expression in total bone marrow cells from healthy donors, and from patients with diagnosis of myelodysplastic syndromes (MDS) stratified by 'WHO (World Health Organization) 2008 classification and acute myeloid leukemia (AML). The "y" axis represents the relative *BNIP3*, *BNIP3L* or *HIF-1α* mRNA expression normalized to *HPRT* endogenous control; sample from a healthy donor was used as the calibration sample. Each dot represents one subject and horizontal lines indicate medians. The numbers of subjects studied and the *P* values (Mann–Whitney test) are indicated. (D) Correlation between *BNIP3* or *BNIP3L* and *HIF-1α* expression in primary bone marrow cells from MDS and AML patients. Spearman correlation test; N, *P* and r values are indicated in the graph. (E) *BNIP3L* mRNA levels in total bone marrow cells from five MDS patients at diagnosis and after disease progression. The "y" axis represents the fold change of *BNIP3L* mRNA expression normalized to *HPRT* endogenous control at diagnosis (calibration sample) and after disease progression; WHO 2008 classification and bone marrow blast percentage are indicated. Data are presented in arbitrary units (a.u.). (F) Overall survival of MDS patients categorized as median of *BNIP3L* expression levels (Kaplan-Meier curves). Patients were subgrouped by higher *BNIP3L* expression (above median; 1.73) and lower *BNIP3L* expression (below median). *P* value is indicated. (G) *BNIP3* and *BNIP3L* methylation in bone marrow samples from healthy donors and from MDS and AML patients. Percentage of positive methylation and total number of samples are indicated. RA: refractory anemia; RARS: refractory anemia with ringed sideroblasts; del(5q): MDS with isolated del(5q); RCMD: refractory cytopenia with multilineage dysplasia; RAEB-1: refractory anemia with excess blasts-1; RAEB-2: refractory anemia with excess blasts-2; MDS: myelodysplastic syndromes; MRC; myelodysplastic-related changes.

still limited.[1] Decitabine is known to act by sequestering DNA methyltransferase 1 (DNMT1), leading to the restoration of silenced genes. However, its direct effects upon cell death may also be important in order to achieve clinical efficacy.[11] In this regard, interventions that potentiate decitabine-induced apoptosis may be beneficial to increase clinical response. CD34+ bone marrow cells of 4 *de novo* AML patients from our cohort were treated with decitabine (0.1, 0.5, 1 and 5μM) for 48 hours. Among these patients, only 1 patient presented *BNIP3* and *BNIP3L* methylation, whereas the others presented no methylation of either gene. Apoptotic cells were measured using annexin V and propidium iodide. Apoptosis was mainly induced with the highest doses of decitabine (1μM and

5μM) and positively correlated with *BNIP3L* expression (r=0.59, *P*=0.006). In contrast, *BNIP3* expression was induced by decitabine only in the methylated sample (*Online Supplementary Figure S1*).

Among a panel of myeloid cell lines, a strong correlation was observed between *BNIP3* and *BNIP3L* expression (r=0.78, *P*=0.01), but only U937 cells presented *BNIP3* hemi-methylation, whereas *BNIP3L* methylation was not detected (Figure 2A,B). *BNIP3* and *BNIP3L* expression was also evaluated in HL60, K562, U937 and HEL cell lines treated with 1mM or 5mM decitabine. *BNIP3L* expression was particularly increased by decitabine treatment in U937 and HEL cells, which presented higher apoptotic levels. *BNIP3* upregulation was observed in U937 cells only, con-
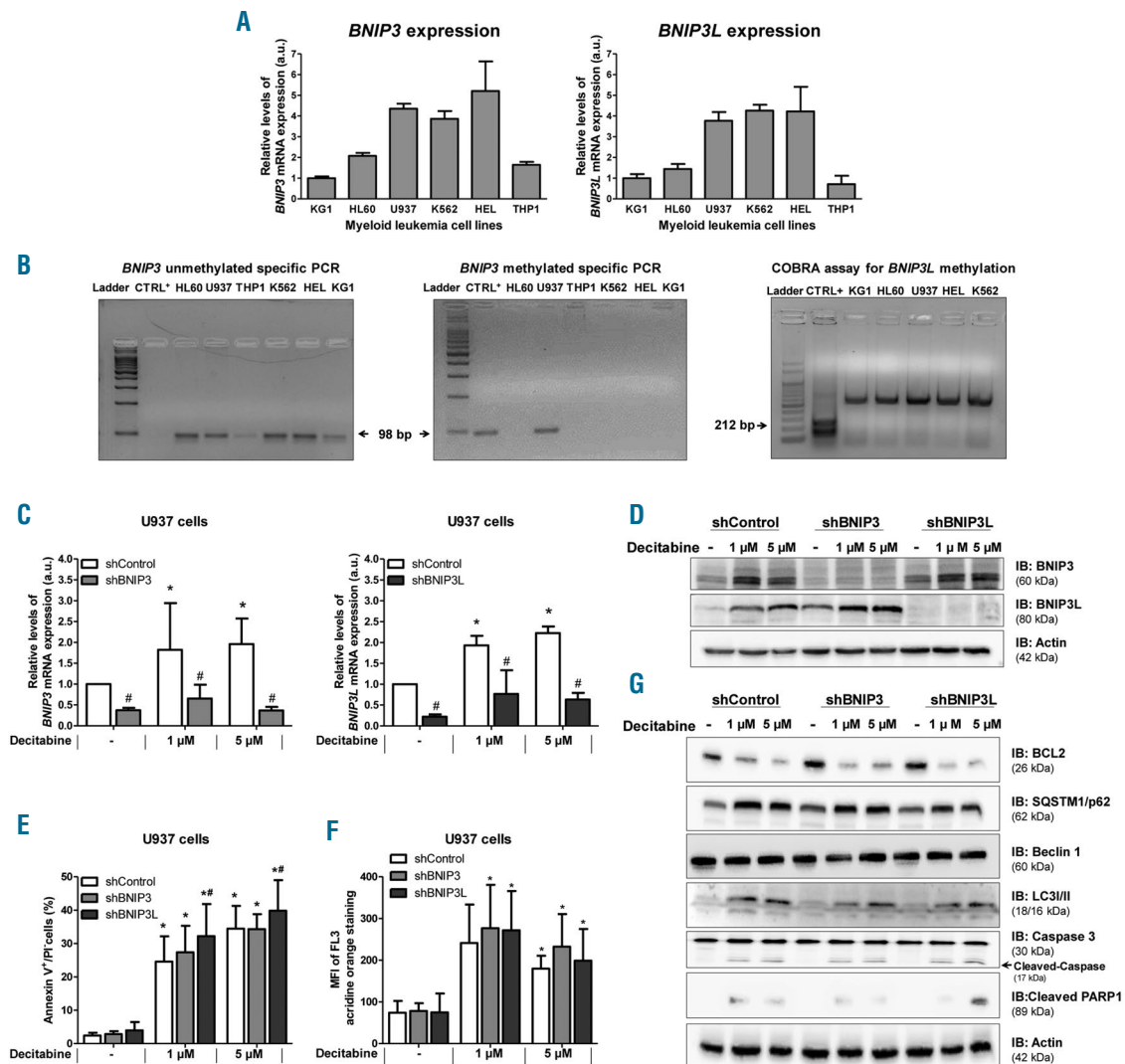

Figure 2. Effects of BNIP3 and BNIP3L silencing on the apoptosis and autophagy of U937 cells treated with decitabine. (A) *BNIP3* and *BNIP3L* mRNA expression in KG1, HL60, U937, K562, HEL and THP1 myeloid leukemia cells. *HPRT* was used as the housekeeping gene and a KG1 cell line was used as the calibration sample. Data are presented in arbitrary units (a.u.). (B) Methylation status of *BNIP3* promoter region in myeloid cell lines evaluated by methylation-specific PCR. Methylation status of *BNIP3L* promoter region in myeloid cell lines evaluated by combined bisulfite restriction analysis (COBRA). For all methylation analyses, a universal methylated human DNA was used as positive control. (C-G) U937 cells were silenced for BNIP3 (shBNIP3), *BNIP3L* (shBNIP3L) or transduced with lentivirus-mediated shRNA control (shControl). Analyses were performed after 48h of decitabine treatment (0, 1 or 5μM). (C) Expression of BNIP3 and BNIP3L was assessed by qPCR (quantitative polymerase chain reaction) and (D) western blot. (E) Apoptosis was detected by flow cytometry using Annexin V/PI staining. (F) Acidic vesicular organelles were detected by flow cytometry using acridine orange staining. All data shown are the mean±SD of 4 independent experiments. *P≤0.05 decitabine-treated cells *vs.* untreated cells under the same conditions, #P≤0.05 shBNIP3L *vs.* shControl cells; Student's *t*-test. (G) Western blot analysis of BCL2, SQSTM1/p62, Beclin 1, LC3I/II expression, caspase 3 (total and cleaved), and cleaved PARP1. The membranes were reprobed with the specific antibody for detection of the respective protein or actin (loading control). PCR: polymerase chain reaction; mRNA: messenger RNA.

comitantly with partial demethylation of its promoter region. Autophagy was evaluated by acridine orange staining and was induced by decitabine in all tested cell lines (*Online Supplementary Figure S2*). Taken together, these results suggest that *BNIP3L* expression is increased by decitabine treatment in myeloid cells that undergo apoptosis, even in the absence of gene promoter methylation.

We next tested whether BNIP3 and BNIP3L could participate in the induction of apoptosis by decitabine. For this purpose, the effects of decitabine treatment (1μM or 5μM for 48h) were investigated in U937 cells silenced for BNIP3 or BNIP3L. Gene silencing was performed using lentivirus-mediated shRNA, as previously described,[9] and was confirmed by quantitative PCR and western blot. In addition to gene expression, protein levels of BNIP3 and BNIP3L were increased by decitabine (Figure 2C,D). Surprisingly, the combination of BNIP3L silencing (but not BNIP3) and decitabine treatment slightly induced apoptosis further (Figure 2E). Decitabine-induced autophagy was not altered by BNIP3 or BNIP3L knockdown in U937 cells (Figure 2F). As expected, decitabine treatment reduced BCL2 expression and increased the expression of SQSTM1/p62, LC3I/II and the cleaved forms of caspase 3 and PARP1. In agreement with annexin V staining, higher levels of cleaved caspase 3 and cleaved PARP1 were detected in U937 cells silenced for BNIP3L and treated with 5μM decitabine (Figure 2G), confirming that BNIP3L silencing and decitabine cooperate to sensitize leukemia cells to apoptosis.

Taken together, we provide the first evidence of *BNIP3* being downregulated in bone marrow cells from MDS patients. *BNIP3L* expression was not only decreased in advanced MDS and AML samples, but was also an independent prognostic factor in MDS. These results are in accordance with BNIP3 and BNIP3L proapoptotic functions, and suggest that BNIP3 is most likely to participate in the imbalance of apoptosis found in MDS cells, whereas BNIP3L may be involved in the decreased apoptosis of high-risk MDS or AML. On the other hand, an essential role of BNIP3L was described during erythroid differentiation.[12,13] BNIP3L knockout mice presented anemia, erythroid hyperplasia and reticulocytosis associated with a critical defect of mitochondrial clearance during reticulocyte maturation.[13] In addition, the failure of mitochondria removal has been proposed to be associated with increased ROS production and subsequent progression to AML.[14] In accordance with this, decreased autophagic flux and increased amounts of defective mitochondria have recently been reported in human AML blasts.[15] Thus, we speculate that the low expression of *BNIP3L* in advanced MDS and in AML may be associated with deficient mitophagy that could favor the progression of these diseases.

The low frequency of methylation in *BNIP3* and *BNIP3L* promoter regions indicates that this process is not the single reason for their decreased expression in patient bone marrow samples. In fact, histone acetylation of the *BNIP3* promoter region was already associated with gene expression in leukemia cell lines.[3] Even when unmethylated, BNIP3L expression was increased in leukemia cells that underwent apoptosis induced by decitabine. Surprisingly, BNIP3L silencing slightly amplified the apoptotic effect of decitabine, contradicting the pro-apoptotic function of this protein. Since autophagy was apparently not modulated, this finding cannot yet be explained. In this sense, further studies will be important to elucidate the participation of BNIP3L in the response of MDS and AML patients to hypomethylating agent-based therapy.

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References

1. Ades L, Itzykson R, Fenaux P. Myelodysplastic syndromes. Lancet. 2014;383(9936):2239-2252.
2. Zhang J, Ney PA. Mechanisms and biology of B-cell leukemia/lymphoma 2/adenovirus E1B interacting protein 3 and Nip-like protein X. Antioxid Redox Signal. 2011;14(10):1959-1969.
3. Murai M, Toyota M, Satoh A, et al. Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumours. Br J Cancer. 2005;92(6):1165-1172.
4. Ripperger T, von Neuhoff N, Kamphues K, et al. Promoter methylation of PARG1, a novel candidate tumor suppressor gene in mantle-cell lymphomas. Haematologica. 2007;92(4):460-468.
5. Bellot G, Garcia-Medina R, Gounon P, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol. 2009; 29(10):2570-2581.
6. Velasco-Hernandez T, Hyrenius-Wittsten A, Rehn M, Bryder D, Cammenga J. HIF-1alpha can act as a tumor suppressor gene in murine acute myeloid leukemia. Blood. 2014;124(24):3597-3607.
7. Vukovic M, Guitart AV, Sepulveda C, et al. Hif-1alpha and Hif-2alpha synergize to suppress AML development but are dispensable for disease maintenance. J Exp Med. 2015;212(13):2223-2234.
8. Xavier-Ferrucio JM, Pericole FV, Lopes MR, et al. Abnormal Hedgehog pathway in myelodysplastic syndrome and its impact on patients' outcome. Haematologica. 2015;100(12):e491-493.
9. Machado-Neto JA, de Melo Campos P, Favaro P, et al. Stathmin 1 is involved in the highly proliferative phenotype of high-risk myelodysplastic syndromes and acute leukemia cells. Leuk Res. 2014;38(2):251-257.
10. Kim TK, Gore SD, Zeidan AM. Epigenetic Therapy in Acute Myeloid Leukemia: Current and Future Directions. Semin Hematol. 2015;52(3):172-183.
11. Voso MT, Santini V, Fabiani E, et al. Why methylation is not a marker predictive of response to hypomethylating agents. Haematologica. 2014;99(4):613-619.
12. Schweers RL, Zhang J, Randall MS, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc Natl Acad Sci USA. 2007;104(49):19500-19505.
13. Sandoval H, Thiagarajan P, Dasgupta SK, et al. Essential role for Nix in autophagic maturation of erythroid cells. Nature. 2008; 454(7201):232-235.
14. Watson AS, Mortensen M, Simon AK. Autophagy in the pathogenesis of myelodysplastic syndrome and acute myeloid leukemia. Cell Cycle. 2011;10(11):1719-1725.
15. Watson AS, Riffelmacher T, Stranks A, et al. Autophagy limits proliferation and glycolytic metabolism in acute myeloid leukemia. Cell Death Discov. 2015;1.